Yeasts and other culturable microorganisms associated with the nickel hyperaccumulator *Berkheya coddii* and its insect herbivore, *Chrysolina clathrata.*

by

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Summary

The heterogeneity and distribution of elements on earth is one of the key drivers that shape the biotic processes in any given environment. What we may consider as anomalies in an environment's element composition often drives biological adaptation and speciation. Serpentine environments provide dramatic examples of the effect soil element composition has on life. The elevated heavy metal concentrations in these environments resulted in the adaptation of plants and insects endemic to these areas. Physiological adaptations of *Berkheya coddii* and its insect herbivore, *Chrysolina clathrata,* allowed them to exploit the nickel (Ni) rich serpentine soil of the Barberton Greenstone Belt. One of the driving forces behind these adaptations may involve interactions with microorganisms. However, the microbiology of serpentine environments is relatively unknown. In the current study we aimed to identify microorganisms that may have symbiotic relationships with *C. clathrata* and its diet, the herbaceous Ni hyperaccumulating plant *B. coddii*. Culture techniques were used to isolate bacteria and fungi from plants and the faeces of beetles that were reared under laboratory conditions. The identity of isolates was determined using morphology and molecular techniques. Several genera of filamentous fungi (*Alternaria, Aspergillus, Bipolaris, Cladosporium, Epicoccum, Fusarium,* and *Penicillium*), yeasts (*Cryptococcus, Meyerozyma,* and *Rhodotorula*), and endophytic bacteria (*Bacillus* and *Lysinibacillus*) were isolated from the leaves of *B. coddii*. Yeast species, representing the genera *Candida, Cryptococcus, Debaryomyces, Meyerozyma* and *Wickerhamomyces* were isolated from faeces of the beetles. The minimum inhibitory concentration (MIC) of Ni was determined for all isolates. The endophytic bacteria, filamentous fungi, *Candida intermedia, Cryptococcus flavescens* and *Meyerozyma guilliermondii* showed notable Ni resistance. The Ni resistant yeast strains were isolated from the faeces of the beetles where the yeasts were in close contact with Ni ions. A strain of *M. guilliermondii* was also found on leaves of *B. coddii* but this strain had lower resistance to Ni and occurred in much lower numbers than the faecal strain. Therefore, it seems that the gut of the beetle selects for Ni resistant yeasts. The role of the yeasts occurring in the gut of the beetle may be to metabolize waste products of the beetle or aid in the sequestration of Ni. Nitrogenous metabolic waste products are usually excreted by terrestrial insects as uric acid and/ or urea. Results obtained by UPLC-MS and colorimetry confirmed that uric acid and urea were present in the faeces of *C. clathrata.* Strains of the yeast species *M. guilliermondii*, *C. flavescens* and *W. anomalus* isolated from the beetle

faeces used uric acid as sole carbon and nitrogen source. A strain of *M. guilliermondii* isolated from the faeces of *C. clathrata* sequestered Ni from an aqueous solution. Concluded from these findings, yeasts in the gut of *C. clathrata,* may play a role in the recycling of nitrogen and may play a role in the reduction of Ni toxicity in the insect.

Keywords: *Berkheya coddii, Chrysolina clathrata,* Ni hyperaccumulation, yeasts, filamentous fungi, endophytic bacteria, serpentine.

Opsomming

Die heterogeniteit en verspreiding van elemente op aarde is een van die sleutel drywers wat die biotiese prosesse vorm in enige gegewe omgewing. Wat ons as abnormaal mag beskou in ‘n omgewing se element samestelling is dikwels die dryfveer van biologiese aanpassing en selfs spesiëring. Serpentynsteen-omgewings verskaf dramatiese voorbeelde van die effek van grond element samestelling op lewe. Die hoë swaarmetaal konsentrasies in hierdie omgewings het gelei tot die aanpassing van plante en insekte tot so ‘n mate dat hul endemies geword het aan die omgewings. Fisiologiese aanpassings van *Berkheya coddii* en sy insek herbivoor, *Chrysolina clathrata,* stel hierdie organismes instaat om die nikkel (Ni) -ryke serpentynsteengrond van die Barberton Groensteen Belt te benut. Een van die dryfkragte agter hierdie aanpassings kan moontlik die interaksies met mikroörganismes wees, maar die mikrobiologie van serpentynsteen-omgewings is relatief onbekend. Die doel van hierdie studie was om mikroörganismes te identifiseer wat simbiotiese verwantskappe kan hê met *C. clathrata* en die insek se voedingsbron, die kruidagtige Ni hiperakkumulerende plant *B. coddii.* Kultuur tegnieke is gebruik om fungi en bakterieë van plante en insekte te isoleer en in die laboratorium aan te kweek. Die identiteit van die isolate is met morfologiese en molekulêre tegnieke bepaal. Verskeie genera filamentagtige fungi (*Alternaria, Aspergillus, Bipolaris, Cladosporium, Epicoccum, Fusarium,* en *Penicillium*), giste (*Cryptococcus, Meyerozyma,* en *Rhodotorula*), en endofitiese bakterieë (*Bacillus* en *Lysinibacillus*) is vanaf die blare van *B. coddii* geïsoleer. Giste verteenwoordigend van die genera *Candida, Cryptococcus, Debaryomyces, Meyerozyma* en *Wickerhamomyces* is ook vanuit die insek se faeces geïsoleer. Die minimum inhiberende Ni konsentrasie van elke isolaat is bepaal. Die endofitiese bakterieë, filamentagtige fungi, *Candida intermedia, Cryptococcus flavescens* en *Meyerozyma guilliermondii* het almal weerstandbiedenheid teen Ni getoon. Die Ni weerstandbiedende gis stamme is vanuit die insek se faeces geïsoleer waar hierdie giste in noue kontak met Ni ione was. ‘n Stam van *M. guilliermondii* is ook op die blare van *B. coddii* gevind maar hierdie stam het laer Ni weerstand gehad en het ook in baie laer getalle voorgekom as die stam wat in die faeces gevind is. Dit wil dus voorkom of die dermkanaal van *C. clathrata* selekteer vir Ni weerstandbiedende giste. Die rol van giste in die dermkanaal van *C. clathrata* kan moontlik wees om afvalprodukte van die insek te metaboliseer of om te help met die sekwestrering van Ni. Stikstofbevattende afvalprodukte, geproduseer tydens stikstof metabolisme, word dikwels deur terresetriële insekte as uriensuur en/of ureum

uitgesky. Resultate van UPLC-MS en kolorimetrie dui op die teenwoordigheid van uriensuur en ureum in die feces van *C. clathrata*. Stamme van die gis spesies *M. guilliermondii*, *C. flavescens* and *W. anomalus* kon uriensuur as ‘n enigste koolstof en stikstofbron benut. ‘n Stam van *Meyerozyma guilliermondii* was in staat om Ni vanuit ‘n waterige oplossing te sekwestreer. Hierdie waarnemings dui daarop dat giste binne die dermkanaal van *C. clathrata* moontlik ‘n rol kan speel in die sirkulering van stikstof en ook bydra tot die verlaging van Ni toksisiteit in die insek.

Sleutelwoorde: *Berkheya coddii, Chrysolina clathrata,* Ni hiperakkumulering, giste, filamentagtige fungi, endofitiese bakterieë, serpentynsteen.

Chapter 1

Literature review

Parts of this chapter were presented at:

1. **The 7th International Conference on Serpentine Ecology, Coimbra, Portugal, 12-16 June 2011.**

2. **The 16th Congress of the South African Society for Microbiology, Cape Town, South Africa, 6–9 November 2011.**

1.1 Background

The Barberton Greenstone Belt (BGB) in the Mpumalanga Province, South Africa, is a remnant of an early Archean orogenic belt with multiple episodes of mafic and ultramafic magmatism and contains serpentine soils rich in heavy metals (HMs) such as nickel (Ni) (Westall et al. 2001). This area hosts a number of endemic plant taxa (Reddy et al. 2001) and one plant species in particular, *Berkheya coddii* Roessler (Asteraceae) (Figure 1.1), has received much attention due to its potential as a phytoremediation and Nickel-phytomining agent (Brooks et al. 1998; Brooks and Robinson 1998; Brooks et al. 2001; Harris et al. 2009; Robinson et al. 1999).

The ecophysiology of *B. coddii* was studied intensively, including its herbivorous grazers and their predators (Augustyniak et al. 2002, 2007; Mesjasz-Przybyłowicz and Przybyłowicz 2001, 2011; Mesjasz-Przybyłowicz et al. 2001, 2002, 2004, 2011; Migula et al. 2007, 2011; Moradi et al. 2010; Robinson et al. 2003). The phytophagous chrysomelid beetle *Chrysolina clathrata* (formerly *Chrysolina pardalina*) (Figure 1.2) was reported for the first time in 1999 (Mesjasz-Przybyłowicz and Przybyłowicz 1999, 2001) when it was shown that this insect developed and proliferated through several generations by feeding exclusively on *B. coddii* leaves. The impact of high Ni concentrations on the various trophic levels of this particular food web has been studied extensively. However, the involvement of microorganisms (including fungi) in this ecophysiological structure, based on ultramafic soil, remains a source of interest.

The aim of this study was firstly to isolate and identify culturable bacteria, filamentous fungi and yeasts associated with *B. coddii* and to determine whether these microorganisms are adapted to high Ni concentrations (Chapter 2). Secondly, we aimed to investigate the possibility of yeasts being present in the gut of *C. clathrata*, and to determine the type of symbiosis that may exist (Chapters 2 and 3). This is the first attempt to understand the interactions of these microorganisms with Ni-hyperaccumulating plants and their insect herbivores.

1.2 Serpentine ecology

Serpentine soils are distributed in patches worldwide and the flora of these ecosystems has been studied since the 1850's (Whittaker 1954). These soils are characteristic in that they do not support high plant productivity, have high rates of endemism and vegetation types distinct from soils of neighbouring areas (Brady et al. 2005; Whittaker 1954;). Three main categories of discussion on the biology of serpentine soils have emerged over the years, i.e. the edaphic, concerning the soil itself and its relation to plant ecology, the plant species-level response (autecology), and the plant community-level effect (synecology) (Whittaker 1954; Brady et al. 2005). Other avenues of serpentine ecology that were explored, albeit to a much lesser extent, are the interaction of microorganisms with serpentine flora and the effect of serpentine microorganisms on higher trophic levels, e.g. herbivores (Lau et al. 2008)

1.3 Edaphic properties of the Barberton Greenstone Belt (BGB)

Soil conditions in terrestrial ecosystems are important determinants both above and underground, affecting the biodiversity of microorganisms, plants and animals (Branco and Ree 2010). The edaphic properties of serpentine soil are influenced by physical, chemical and biotic factors (Brady et al. 2005).

The BGB in the Mpumalanga Province of South Africa is 3.4-3.2 Ga old (Van Zuilen et al. 2007) and is a remnant of an early Archean orogenic belt (Westall et al. 2001). The geology of this area is very complex due to various processes that were involved in creating the rocks. Multiple episodes of mafic (silicate mineral that is rich in magnesium and iron) and ultramafic magmatism, syntectonic tonalite intrusion and eruption of equivalent rocks, fold and thrust deformation, syntectonic sedimentation and hydrothermal processes played an important role in shaping of these rocks (Van Zuilen et al. 2007; Westall et al. 2001). Common minerals associated with ultramafic soils are antigorite, quartz, chlorine and olivine (Terlizzi and Karlander 1979), but the chemical composition of ultramafic rocks varies greatly. Weathering of these rocks creates serpentine soils which are characterized by high concentrations of HM's, especially Ni (400-14 000 $mg.kg^{-1}$), high Mg:Ca ratios and low concentrations of nitrogen, phosphorus and potassium (McGrath 1995; Reddy et al. 2009). These chemical

characteristics create harsh environmental conditions that result in low plant diversity and unique, usually endemic, flora (Reddy et al. 2009).

The high Ni concentrations in the BGB serpentine soil is especially harsh, since although this HM is an essential element required for normal growth and metabolism of bacteria and plants, excessive amounts can be cyto- and phytotoxic (Abou-Shanab et al. 2007; Rascio and Navari-Izzo 2011). The mechanisms of Ni-toxicity include alterations in physiological processes on a cellular or molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting essential elements and disrupting membrane integrity (Rascio and Navari-Izzo 2011). In addition, Ni is known to be a carcinogen, especially in ionic form, Ni^{2+} (Congeevaram et al. 2007). Interestingly, Ni is considered an evolutionary relic of a pre-oxygen era when it was used to metabolise chemicals such as methane, carbon monoxide and hydrogen (Crichton 2008). This is reflected in the abundance of this metal in a number of anaerobic bacteria. In contrast, the level of Ni in mammalian serum is 100- fold less compared to zinc, iron and copper (Chen and Wong 2006). Nickel containing proteins are virtually unknown in higher eukaryotes, with the exception of the plant enzyme, urease (Abou-Shanab et al. 2007; Rascio and Navari-Izzo 2011).

The physical properties of serpentine soils also contribute to the inhospitable conditions. Outcrops exist that are often steep and comparatively rocky making them particularly vulnerable to erosion, which results in shallow soils (Brady et al. 2005). Moreover, silt and clay contents in serpentine soils are generally minimal. The combination of these characteristics yields an environment with relatively low nutrient levels and little moisture (Brady et al. 2005).

Biotic interactions, such as competition and herbivory, may limit plant species ranges to a subset of edaphically suitable habitats, known as the realized niche. For example, Lau and co-workers (2008) demonstrated that edaphic environmental variables impacted ecotypes of *Collinsia sparsiflora* (endemic to serpentine areas in California) directly and indirectly by altering interactions with herbivores. Similarly, Pollard and Baker (1997) showed that hyperaccumulation of Zn by *Thlaspi caerulescens* prevented generalist herbivory. Differential

feeding of herbivorous insects on plants with lower HM concentrations could result in selection pressures favouring the evolution of hyperaccumulation (Pollard and Baker 1997). Plant pathogens could have excerted a similar selective pressure (Brady et al. 2005). Therefore, edaphic factors may affect plant traits that, in turn, alter their attractiveness to herbivores and their susceptibility to pathogens.

1.4 Synecology of hyperaccumulators endemic to the BGB

All ecological communities are the products of biogeographic history, the physical environment, and biotic interactions (Harrison et al. 2007). Arguably, the most important factor impacting on plant communities is the soil properties. Ecotypic variation in plants is a response to diverse environments and soils derived from ultramafic rocks can act as an agent of ecotypic selection (Reddy et al. 2001). In any given locality, indifferent or *bodenvag* taxa occur both on serpentine and adjacent non-serpentine soils. These taxa may be taxonomically identical, but may be ecotypically differentiated. Generally, the more stressful the sites, the less common are *bodenvag* taxa (Reddy et al. 2009).

Whether *B. coddii* (Figure 1.1) is a *bodenvag* taxon still needs to be evaluated during future surveys. However, populations of this plant, which are geographically separated between different serpentine sites, differ in their ability to hyperaccumulate Ni (Mesjasz-Przybyłowicz et al. 2004). Interestingly, studies done by Mesjasz-Przybyłowicz and co-workers (1997) on another Ni hyperaccumulator, *Senecio coronatus,* which occurs on the same serpentine outcrops as *B. coddii,* showed the existence of three genotypes of *S. coronatus.* These genotypes were geographically separated with two of these populations hyperaccumulating Ni whilst the third population had lower Ni concentrations, below the hyperaccumulation threshold of 1000 $\mu g.g^{-1}$.

1.5 Autecology of *Berkheya coddii*

In the presence of elevated toxic HM concentrations, terrestrial plant species can develop two basic strategies to protect themselves: exclusion or accumulation of metals (Montargès-Pelletier et al. 2008). Non-accumulating plants maintain metals at relatively low concentrations within their vital tissues by avoiding excessive metal uptake and transport. In

contrast, metal-tolerant plant species absorb metals through their roots and translocate them to aerial parts e.g. stems and leaves (Montargès-Pelletier et al. 2008). Plants that are capable of accumulating HM at concentrations above 1000 $\mu g.g^{-1}$ dry matter are known as HM hyperaccumulators (Brooks et al. 1977). This is an extremely rare and intriguing phenomenon (Montargès-Pelletier et al. 2008). To date there are only about 400 known hyperaccumulating species (Robinson et al. 1997), of which 318 are Ni hyperaccumulators (Mesjasz-Przybyłowicz et al. 2004).

Figure 1.1: Flowering *Berkheya coddii* (Photo: F. Postma; Fujifilm HS 10 digital camera)

The physiological adaptation mechanisms of *B. coddii* towards high Ni concentrations have been the subject of many studies due the phytomining and phytoremediation applications of this plant (Aggarwal and Goyal 2007; Brooks and Robinson 1998; Mesjasz-Przybyłowicz et al. 2004; Rascio and Navari-Izzo 2011; Robinson et al. 1997; Salt et al. 1998). The success of phytoremediation depends on the ability of a plant to accumulate concentrations of heavy metals in shoots high enough to reduce the soil concentration of such metals to regulatory levels with relatively few harvests (Do Nascimento et al. 2006). Mesjasz-Przybyłowicz and

co-workers (2004) reported an average Ni concentration of 17 900 $\mu g.g^{-1}$ Ni in leaves of *B. coddii* collected from a mining site. The phytoextraction coefficient (PC), i.e. the ratio between µg metal/g dry weight of tissue and µg metal/g dry weight of substrate, was 13.63. The same authors reported Ni concentrations of 28 200 $\mu g.g^{-1}$ (PC = 21.48) in leaves from young plants and 19 700 $\mu g.g^{-1}$ (PC = 15.00) in leaves from mature vegetative shoots. The highest concentration of Ni accumulated by *B. coddii* (54 600 ± 1500 $\mu g.g^{-1}$, with the highest sample reaching 76 100 $\mu g.g^{-1}$) was reported in leaves collected at another ultramafic location. The Ni concentration in the soil near the plant roots showed a typical value for ultramafic soil, i.e. 1 280 $\mu g.g^{-1}$ (Mesjasz-Przybyłowicz et al. 2004).

By using *B. coddii* for phytoremediation, biomass of 22 t dry weight per ha can be obtained after moderate fertilization without a decrease in nickel concentration within the plant tissue. This renders the plant ideal for remediation of Ni contaminated soil by means of phytoextraction (Moradi et al. 2010). Presently, it is cultivated commercially in South Africa (Rustenburg Base Metals Refiners) and has also been tested for phytoextraction in New Zealand and the United States. The feasibility of recovering nickel and producing biofuels from nickel-containing biomass of *B. coddii* has also been investigated in Japan (Mesjasz-przybyłowicz et al. 2004).

The cellular and sub-cellular localization of Ni accumulated in *Berkheya coddii* has been analyzed using either chemical extractions of Ni from separated organs of plants, electron microscopy (SEM, TEM) or nuclear microscopy (PIXE) coupled with microanalysis (Montargès-Pelletier et al. 2008). The highest concentrations of Ni were found in the leaf margins, leaf mesophyll, and leaf epidermis with a maximum in the cuticle of upper epidermis (Gramlich et al. 2011; Mesjasz-Przybyłowicz et al. 2001; Montargès-Pelletier et al. 2008; Robinson et al. 2003). Biochemical pathways involving Ni transport and storage were shown to be physically separated from those of Ca and Mn. Also, cells with higher vacuolar volume appear to be preferential accumulation sites and vacuolar Ni storage in leaf cells appears as the main biochemical detoxification mechanism (Montargès-Pelletier et al. 2008).

The low solubility and reactivity of metal ions at physiological pH suggest that chelation mechanisms must be operative once metals are taken up into the cell, and in particular within

vacuoles. The main classes of metal chelators in plants are phytochelatins (peptides, oxygen and nitrogen donor ligands) and metallothioneins (sulphur donor ligands), organic acids (oxygen donor ligands), amino acids (oxygen and nitrogen donor ligands) and other high molecular weight molecules (proteins, chaperones). Histidine, citrate and malate are the most frequent ligands for Ni, Zn and Cd in the aerial parts of plants (Montargès-Pelletier et al. 2008)

1.6 *Chrysolina clathrata*

It was hypothesized that *B. coddii* and other hyperaccumulators accumulate HMs to deter herbivorous insects and pathogenic microorganisms (Chen and Wong 2006; Rascio and Navari-Izzo 2011). However a few insect species, also endemic to ultramafic environments, have evolved parallel with these plants and are consequently specialized herbivores of hyperaccumulators (Mesjasz-Przybyłowicz and Przybyłowicz 2001; Augustyniak et al. 2002, 2007). Never the less, the existence of such adapted insects suggests that Ni-hyperaccumulation can benefit the plants in terms of defence against the majority of herbivores. In addition, it is strongly suspected that hyperaccumulating plants elaborate allelopathy strategies by increasing local Ni phytoavailability in their immediate vicinity, through the deposition of Ni-rich senescent leaves (Boyd and Martens 1992, 1994; Montargès-Pelletier et al. 2008). However the herbivorous insect *Chrysolina clathrata* (Figure 1.2) is not deterred by high Ni concentrations (Boyd et al. 2009).

The insect genus *Chrysolina* (Coleoptera: Chrysomelidiae: Chrysomeliniae) is extensive and diverse, containing specialized phytophagous beetle species (Bieñkowski 2001). They feed on eight plant families with the Asteraceae and Lamiaceae most frequently selected (Garin et al. 1998). To date 65 subgenera, and almost 450 species, have been identified which are mainly distributed in Europe, Asia and Africa (Bieñkowski 2001). However, due to their specialized feeding behaviour certain species have been introduced as biocontrol agents of St Johnwort (*Hypericum perforatum*) in the United States (Cambell and McCaffrey 1991), and New Zealand (Paynter et al. 2002). Also, species of *Chrysolina* endemic to South Africa are currently used to control the invasion of Bitou bush (*Chrysanthemoides monilifera* spp. *rotundata*) and boneseed (*Chrysanthemoides monilifera* spp. *monilifera*) in Australian

conservation areas (Scott and Adair 1990). To date, 37 endemic *Chrysolina* species have been found in South Africa (Bieñkowski 2001; Scott and Adair 1990).

Insects often have unusual or restricted diets (Gullan and Cranston 2010) and *Chrysolina clathrata* (formerly *C.* pardalina) is no exception. This chrysomelid beetle adapted to an environment rich in Ni and has been shown to complete its entire life cycle for several generations feeding exclusively on leaves of *B. coddii* (Mesjasz-Przybyłowicz and Przybyłowicz 2001; Boyd et al. 2009). Interestingly, this beetle flourishes on a diet rich in Ni at concentrations (40 000 - 76 000 µg/g) generally regarded as toxic to most organisms (Mesjasz-Przybyłowicz et al. 2004). This beetle is able to survive this toxicity due to adaptive physiological mechanisms, particularly of the Malpighian tubules and the midgut, whereby excess Ni is sequestered mainly in the faeces and to a lesser extent through the exuviae during moulting of the cuticula (ecdysis) (Migula et al. 2011, Przybyłowicz et al. 2003).

Figure 1.2: Adult *Chrysolina clathrata* beetle feeding on *Berkheya coddii* leaf. (Photo: F. Postma; Nikon SMZ 10A Stereomicroscope at 10x magnification, equipped with a Nikon Coolpix 990 digital camera)

The Malpighian tubules form part of the cryptonephridial excretory system of *C. clathrata,* which contains six pairs of tubules playing a central role in the sequestration of Ni from the insect. The Malpighian tubules are responsible for osmotic regulation, excretion of metabolic waste products and the removal of heavy metals from the haemolymph (Gullan and Cranston 2010). Not surprisingly, the Malpighian tubules are the organs with the highest Ni concentration (Przybyłowicz et al. 2003).

Cations and anions enter the Malpighian tubules using different mechanisms. Also, the types of ions that can be transported can vary greatly depending on the insect species and the individual's physiological needs and demands (Gullan and Cranston 2010). The Malpighian tubules of *C. clathrata* are rich in K^+ that activates a chlorine pump, creating a high osmotic pressure. This not only reduces water loss but also intensifies the reabsorption of metal ions from the haemolymph. Calcium ion channels present in the Malpighian tubules may also ease the transport of Ni^{2+} between the cells through a co-transport mechanism (Migula et al. 2011). Moreover, the interaction of Ni and Zn ions in the Malpighian tubules may contribute to the Ni resistance of this insect. Migula and co-workers (2011) proposed that Ni may replace Zn within the epithelial cells of the tubules. Zinc is a cofactor of many enzymes and is usually bound to S- or N- bearing ligands of low-molecular weight proteins present in the Malpighian tubules. Maintaining a high Zn concentration may thus reduce the toxicity of Ni, which has a similar atomic radius to Zn, by outcompeting Ni for active sites (Migula et al. 2011). A combination of all these ionic mechanisms allows for the effective sequestration of Ni from the beetle.

In addition to the above mentioned physiological adaptations of the Malpighian tubules, structural and cellular adaptations of these tubules and the gut contribute greatly to the sequestration of Ni in *C. clathrata*. The apical part of the Malpighian tubules tightly surrounds the foregut, accumulating the highest Ni concentration (> 3500 µg $Ni.g^{-1}$) while the proximal part (< 35 µg $Ni.g^{-1}$) reaches the midgut into which the contents of the tubules are released (Migula et al. 2011). The highest concentration of Ni is accordingly found in the midgut (Przybyłowicz et al. 2003). As much as 66 % to 75 % of the total heavy metal content of insects feeding on HM hyperaccumulating plants are found in the guts of insects (Boyd et al. 2009). Moreover, in *C. clathrata* there is direct spatial contact between the Malpighian

tubules and the midgut epithelial cells (Migula et al. 2011), which may enhance the sequestration of Ni. This system is highly specific for Ni and the addition of lead or cadmium to the beetle's diet, significantly decreases the sequestration of Ni from the insect (Migula et al. 2011). All these adaptations allow *C. clathrata* to occupy a niche that is potentially toxic for many organisms.

1.7 Serpentine microbial ecology

1.7.1 Microorganisms in serpentine soil

Many essential functions for a sustainable biological community are mediated by soil microorganisms, including bioweathering, nutrient cycling, soil structure, and biological interactions (De Grood et al. 2005). Physiologically stressful environments tend to host depauperate and specialized biological communities. Serpentine soils exemplify this phenomenon by imposing well-known constraints on plants; although their effect on other organisms is still poorly understood (Branco and Ree 2010).

When studying the influence of edaphic properties on serpentine soil microorganisms, De Grood and co-workers (2005) found that neither the Ca and Mg concentrations nor the Ca:Mg ratio of the soil explained the variation in microbial community patterns. However, they established that the organic matter and K levels in soil had the most significant influence on the response of microorganisms, probably because of the associated increase in nutrient availability and water holding capacity.

Serpentine soil bacterial communities tolerate spiking of the soil with metals such as Ni and Zn, more than those of nonserpentine soils (Abou-Shanab et al. 2007). Evidence also exist that soil near hyperaccumulating plants contains a greater proportion of bacteria with metal resistance (Abou-Shanab et al. 2007; Ma et al. 2009). Moreover, the addition of HMs to serpentine soil was found to cause only minor shifts in soil microbial communities compared to nonserpentine soils, suggesting that the serpentine microbial community has greater adaptations to higher HM conditions than nonserpentine microbial communities (De Grood et al. 2005). The total microbial mass is also significantly lower in serpentine than nonserpentine soil (De Grood et al. 2005). Heavy metals influence microbial populations by

affecting their growth, morphology and biochemical activities, ultimately resulting in decreased biomass and diversity (El-Meleigy et al. 2011). Microbial survival in HM containing soils, therefore, depends on intrinsic biochemical and structural properties, physiological and/or genetic adaptation, including morphological changes of cells and environmental modification of the HM's chemical state referred to as metal speciation (Abou-Shanab et al. 2007; El Meleigy et al. 2011).

Microorganisms apply various types of resistance mechanisms in response to HMs. These mechanisms may be encoded by chromosomal genes, although loci conferring resistance are more often located on plasmids (Abou-Shanab et al. 2007). Microorganisms may directly reduce many highly toxic metals via detoxification pathways, thereby reducing the mobility and toxicity of these metals. Five basic mechanisms convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell, (2) enzymatic conversion, (3) intra- or extracellular sequestration, (4) exclusion by a permeability barrier and (5) reduction in sensitivity of cellular targets (El Meleigy et al. 2011).

Various classes of microorganisms have been studied in relation to their role in serpentine environments. The stability and genesis of soil aggregates are directly linked to the clay mineralogy and dissolution processes, as well as the presence of binding factors such as plant root exudates, fungal hyphae and extracellular polysaccharides produced by photosynthetic cyanobacteria (Mapelli et al. 2012). Lichens have been implicated in performing a critical function during the initial stages of serpentine soil formation (Favero-Longo et al. 2005). Terlizzi and Karlander (1979) reported the presence of soil algae from a Maryland U.S.A serpentine formation. The algal flora consisted of members of the Cyanophyta, Chlorophyta and Chrysophyta (Bacillariophyceae). On a divisional level, this soil flora was similar to that of more favourable soil types, and it was suggested that these particular algae have adapted to the serpentine soil conditions. Among the Cyanophyceae occurring in the serpentine soil, three genera (*Scytonema, Gleocapsa, Anabaena*) were found that are known to contain N_2 fixing species, suggesting a potential avenue whereby N could flow into this otherwise nitrogen-poor ecosystem (Terlizzi and Karlander 1979).\

The relative proportion of actinomycetes, compared to other soil microorganisms, is significantly greater in serpentine than in nonserpentine soils (De Grood et al. 2005). Actinomycetes are well known for their ability to adapt to high concentrations of HMs, which explains the high prevalence of these organisms in serpentine soils. The limited available data suggests that microbial and fungal biomass as well as community structure differ between serpentine soils and adjacent nonserpentine soils (De Grood et al. 2005). Interestingly, bacteria are important for Ni hyperaccumulation and may potentially be utilized as an inoculum for enchanced uptake of Ni during commercial phytoremediation (Abou-Shanab et al. 2006).

1.7.2 Microorganisms associated with HM hyperaccumulating plants

1.7.2.1 Rhizosphere

Despite similarities to nonaccumulating plant species, the rhizosphere of hyperaccumulators may have unique properties (Alford et al. 2010). Unfortunately, a general lack of ecological knowledge exists with respect to the rhizosphere processes of hyperaccumulators. Plant performance in serpentine soil is often affected by communities of soil bacteria and fungi. Rhizospheric microorganisms enhance host plant growth by various mechanisms, including atmospheric nitrogen fixation, solubilisation of phosphate, or the production of plant growth regulators (Ma et al. 2009; Doubková et al. 2012). Naturally occuring metal resistant rhizosphere microorganisms can also affect trace metal mobility and availability to the plant through the release of siderophores and chelating agents, acidification, sulphide precipitation and redox changes (Idris et al. 2004; Ma et al. 2009).

A study by Abou-Shanab and co-workers (2006) showed that bacterial species belonging to the genera *Microbacterium, Rhizobium, Clavibacter,* and *Acidovorax*, isolated from the rhizosphere of the Ni hyperaccumulator *Alyssum murale,* increase the phytoavailability of Ni in soils and thereby enhance Ni accumulation. Later, Pal and co-workers (2007) studied the microbial rhizosphere community of two Ni hyperaccumulators, *Rinorea bengalensis* and *Dichapetalum gelanioides,* and found it to be a habitat with a relatively low microbial density that is dominated by bacteria. The Ni minimum inhibitory concentrations (MIC) of bacterial isolates originating from this habitat ranged from 13.6 to 28.9 mM Ni. The metal resistant (> 8

mM Ni) bacteria represented the genera *Bacillus, Cupriavidus* and *Pseudomonas,* and were more tolerant to Ni than fungi originating from the same habitat. However, despite these findings fungi occuring in the rhizosphere are known to play an important role in the growth and survival of hyperacummulating plants.

Various authors have described the beneficial symbiotic interactions of arbuscular mycorrhizal fungi (AMF) that contribute to the edaphic stress tolerance of hyperacummulating plants (Doherty et al. 2008; Doubková et al. 2012; Turnau and Mesjasz-Przybyłowicz 2003). *Berkheya coddii* and other Ni hyperaccumulating members of the Asteraceae family growing in the BGB are also associated with arbuscular mycorrhizal fungi (Turnau and Mesjasz-Przybyłowicz 2003). Moreover, improved Ni accumulation and shoot biomass were observed when *B. coddii* was reared in serpentine soil, heavily inoculated with *Glomus intraradices* (Turnau and Mesjasz-Przybyłowicz 2003).

1.7.2.2 Endophytes

Similar to rhizosphere microorganisms, endophytes have an intimate relationship with their host and have to tolerate high levels of heavy metal concentrations when colonizing hyperaccumulating plants (Idris et al. 2004). Endophytic bacteria enhance plant growth, and increase plant resistance to pathogens, heavy metals, drought and herbivores (Idris et al. 2004; Rajkumar et al. 2009). This prompted researchers to investigate the endophytes of hyperaccumulators in search for novel and promising biosorbents and ways to increase the effectiveness of phytoremediation (Sheng et al. 2008; Xiao et al. 2010). Hyperaccumulating plant species are hosts to a great deal of endophytic biodiversity (Table 1.1). However, no literature is available on the existence of endophytes in the aerial parts of *B. coddii.*

Heavy metal hyperaccumulator endophytes belong to a wide range of phylogenetically unrelated bacterial taxa (Table 1.1). These include *Arthrobacter, Bacillus, Clostridium, Curtobacterium, Enterobacter, Leifsonia, Microbacterium, Micrococcus, Paenibacillus*, *Pseudomonas, Staphylococcus, Stenotrophomonas, Sanguibacter* and *Xanthomonadaceae* (Barzanti et al. 2007; Li et al. 2012). In contrast, only one reported case exists of an endophytic filamentous fungus (*Microsphaeropsis* sp.) associated with a HM

hyperaccumulating plant (Xiao et al. 2010). However, several fungal genera, including *Alternaria, Aspergillus, Mucor, Phoma, Peyronellaea* and *Steganosporium* were found in HM resistant plants, but their life strategies in relation to these plants are unknown (Li et al. 2012).

In general, endophytes enter plant tissue through the root zone, flower, leaf, stem or cotyledon and they may either become localized at the point of entry or spread throughout the plant. Endophytes colonize a niche similar to that of pathogens but they do not cause damage to the plant (Idris et al. 2004) and may be obligate or facultative in their life strategies. Obligate endophytes are strictly dependant on the host plant for growth and survival, and are transmitted vertically or via vectors to other plants. Facultative endophytes have a life cycle stage in which they exist outside host plants (Li et al. 2012). The localization of an endophyte within a particular plant organ or species has been found to influence the HM sensitivity of microbial strains of the same species (Idris et al. 2004; Li et al. 2012). This suggests that long term exposure to different levels of HMs in plant tissues leads to different metal tolerances and adaptations of microorganisms and possibly plays a role in speciation.

Table 1.1: Overview of endophytes associated with hyperaccumulating plants and their occurence in plant organs

Hyperaccumulators	Heavy metal accumulated	Endophytes	Plant organ/s	References
Alyssum bertolonii	Ni	*Arthrobacter*	roots	Barzanti et al. (2007)
		Bacillus	roots	
		Curtobacterium	roots, stems	
		Leifsonia	roots	
		Microbacterium	roots, stems, leaves	
		Pseudomonas	roots, stems, leaves	
		Staphylococcus	roots, stems, leaves	
Alnus firma	Pb, Cu	*Bacillus* sp.	roots	Shin et al. (2012)
Brassica napus	Pb	*Microbacterium* sp.	roots	Sheng et al. (2008)
		Pseudomonas fluorescens	roots	
Nicotiana tabacum	Cd	*Clostridium aminovalericum*	seeds	Mastretta et al.(2009)
		Enterobacter sp.	seeds	
		Pseudomonas fulva	seeds	
		Pseudomonas sp.	seeds	
		Sanguibacter sp.	seeds	
		Stenotrophomonas sp.	seeds	
		Xanthomonadaceae	seeds	
Thlaspi caerulescens	Zn, Cd	*Sphingomonas* sp.	stems	Lodewyckx et al. (2002)
		Methylobacterium sp.	stems	
		Spingobacterium multivorum	stems	
		Phyllobacterium sp.	roots	
		Devosia sp.	roots	
		Sphingomonas sp.	roots	

Table 1.1: continued

		Rhodococcus sp.	roots	
		Afibia sp.	roots	
Thlaspi goesingense	Ni	*Bacteroides*	roots, stems	Idris et al. (2004)
		Cytophaga	roots, stems	
		Flexibacter	roots, stems	
		High G+C gram positive	roots	
		Holophaga acidobacterium	roots, stems	
		Low G+C gram positive	stems	
		Methylobacterium mesophilicum	roots, stems	
		Okibacterium	roots	
		Rhodococcus	roots	
		Sphingomonas spp.	stems	
		Verucomicrobia	roots	
		α-Proteobacteria	roots, stems	
Solanum nigrum	Cd	*Bacillus*	roots, stems, leaves	Guo et al. (2010)
		Microsphaeropsis sp. (fungus)	Stems	Xiao et al. (2010)
		α-, β-, γ-Proteobacteria	roots, stems, leaves	Luo et al. (2011)
		Agrobacterium tumefaciens	roots	
		Arthrobacter oxydans	roots, stems	
		Arthrobacter sp.	roots, stems	
		Bacillus sp.	roots, leaves	
		Bacteroidetes	roots, stems, leaves	
		Chryseobacterium sp.	stems, leaves	
		Curtobacterium sp.	roots, stems, leaves	
		Flavobacterium sp.	stems, leaves	

Table 1.1: continued

	Microbacterium foliorum	roots, stems, leaves	
	Microbacterium hydrocarbonoxydans	roots, stems, leaves	
	Microbacterium sp.	roots, stems, leaves	
	Pseudomonas oryzihabitants	roots, stems	
	Serratia marcescens	roots, stems, leaves	
	Serratia sp.	roots, stems, leaves	
	Sphingomonas sp.	stems	
	α-, β-, γ-Proteobacteria	roots, stems	Chen et al. (2012)
	Actinobacteria	roots, stems	
	Bacteroidetes	roots, stems	
	Firmicutes	roots, stems	

1.7.2.3 Epiphytes and pathogens

Microbial isolates representing at least 78 phylogenetically unrelated bacterial species, and several hundred species of fungi, including plant pathogens, have been isolated from the surfaces of many plant species (Andrews and Harris 2000). However, the microbial epiphytic community of hyperaccumulating plants, comprising a diverse group of micro- and occasionally macroepiphytes (lichens, bryophytes and algae), as well as plant pathogens, generally remains poorly described.

It is well known that plants are continually engaged in a co-evolutionary struggle for dominance with their pathogens (Dodds and Rathjen 2010). The outcomes of these interactions between pathogens and their hosts can have dramatic effects on human activities, for example filamentous microorganisms from the kingdoms Fungi and Stramenopila cause many destructive crop diseases. Plants are exploited by these pathogens, which extract nutrients from the living cells of their hosts (McDowell 2011). These microorganisms are a versatile group which can adapt and grow at relatively extreme conditions of temperature, pH, nutrient availability and high HM concentrations (Ezzouhri et al. 2009). To our knowledge, no studies on the physiology of Ni-hyperaccumulator plant fungal pathogens have been done. The co-evolution of such pathogens with their hosts may have significant implications for phytoremediation efforts.

One possible mechanism by which a fungal pathogen may survive the high Ni concentrations within its Ni-hyperaccumulating host is via sequestration of the HM. It is known that much of the ability of fungi to sequestrate HMs is situated in the fungal cell wall, which contains a variety of functional groups involved in metal binding, and comprises a substantial percentage of fungal biomass (Dhankhar and Hooda 2011). Fungal biomass has consequently received much attention as potential biosorbent and it was found that strains, known to sequestrate Ni, belong to the filamentous fungal genera *Aspergillus, Penicillium, Mucor, Rhizopus,* as well as to the yeast species *Saccharomyces cerevisiae* and *Candida tropicalis* (Dhankhar and Hooda 2011). Although yeasts are known to sequestrate Ni through various mechanisms (Breierová et al. 2008), there is no literature on the occurrence or the role of yeasts in environments with naturally high concentrations of Ni.

Fungal epiphytic communities consist mainly of yeast populations and rapidly sporulating filamentous fungi belonging to genera such as *Alternaria, Cladosporium, Epicoccum, Microsphearopsis, Cytospora* and *Dendrophoma* (Andrews and Harris 2000). Phylloplane yeasts are traditionally broadly grouped into "pink" and "white" forms. Pink yeasts include primarily basidiomycetous species belonging to the genera *Rhodosporidium, Rhodotorula* and *Sporobolomyces,* while white yeasts belong to the genus *Cryptococcus* (Andrews and Harris 2000; Fonseca and Inácio 2006). Another general trend observed during surveys of culturable yeasts occurring on leaf surfaces, is that these yeast populations are commonly dominated by relatively few species, a situation also observed for other microbial epiphytes (Fonseca and Inácio 2006). Amongst the dominant yeast species *Cryptococcus laurentii, Cryptococcus albidus, Rhodotorula glutinis, Rhodotorula minuta, Rhodotorula mucilaginosa* and *Sporobolomyces roseus* appear to be prevalent regardless of plant type or geography. Ascomycetous yeasts are usually rare on the phylloplane although strains representing *Debaromyces hansenii* has been found on various plant species around the world (Fonseca and Inácio 2006). It was found that epiphytic yeasts tend to be active phylloplane colonizers, whereas the filamentous fungi are mostly transients existing on leaves as dormant spores (Andrews and Harris 2000). Extensive epiphytic hyphal growth on healthy, intact, non-senescent leaves is relatively rare.

Microbial phylloplane colonists are presumably endowed with phenotypes suitable for survival and/or growth in their particular surface habitats (Fonseca and Inácio 2006). These niche specific traits may include fast growth rates, the ability to compete for nutrients and to withstand periods of drought or intense light, varying nutrient levels, osmotic conditions and temperatures (Andrews and Harris 2000; Fonseca and Inácio 2006). Additionally, epiphytes of hyperaccumulating plants may have to contend with HM toxicity.

1.8 Yeasts associated with leaf feeding beetles

There are many interactions between fungi and insects ranging from obligate to transient associations. Some of these kill the insects, although a large number benefits either the insect or the fungus or the benefit is reciprocal (Blackwell 2010). In our study, microorganisms residing within the gut of *C. clathrata* are of special interest, since they presumably come in

contact with high concentrations of Ni. The digestive tract of these phytophagous beetles presents a largely unexplored habitat for many microorganisms including filamentous fungi and yeasts (Nguyen et al. 2006).

Insects that feed on plants and fungi are often associated with yeasts (Suh et al. 2008) and in some cases, yeast clades or metabolic guilds have specific associations with certain insect species (Suh et al. 2004). Ascomycetous yeasts are often found in the insect gut, sometimes as endosymbionts in special compartments (Blackwell 2010). The majority of yeast endosymbionts are classified as species of true yeasts belonging to the genus *Candida* (asexual Saccharomycetales) (Zhang et al. 2003).

Yeasts associated with phytophagous insects detoxify plant metabolites or provide the necessary enzymes to attack plant cell walls that are intractable to insects. Many insects rely on microbial enzymes for the fermentation of food resources to improve the nutritional content thereof (Blackwell 2010; Nguyen et al. 2006). These interactions allow insects to occupy new habitats, at relatively little genetic expense (Blackwell 2010). However, direct relationships between fungi and insect evolution remain poorly studied (Suh et al. 2004). The gut microbiota of *C. clathrata* may have an additional role in the sequestration of Ni from the insect. Yeasts adsorb various heavy metals, including Ni (Brady et al. 1994; Padmavathy 2007) and numerous studies have focused on the application of yeasts as biosorbents of heavy metals in polluted environments. This adsorption capability of yeasts may effectively reduce the free Ni ion concentration in the gut of *C. clathrata* therefore limiting the toxic effect of this metal. For this sequestration mechanism to be viable and provide continuous protection, the yeast community in the gut should be relatively stable. Extracellular symbiotic communities in the gut are, however, vulnerable to invasion of foreign microorganisms and are therefore thought to be evolutionary unstable. Host-symbiont co-speciation and reductive genome evolution is thus rarely observed among extracellular insect symbionts such as those found in the digestive tract (Kikuchi et al. 2009). The gut of the insect may, however, create a selective environment were certain yeast species tend to be dominant.

The culturable yeasts species diversity in the gut of an insect is usually very low, often comprising of a single species (Zhang et al. 2003). Also, the degree of host specificity of the

yeasts was found to vary depending on the beetle species (Zhang et al. 2003). Molnár and co-workers (2008) isolated representatives of several yeast genera from the gut of the maize pest *Diabrotica virgifera* (Coleoptera: Chrysomelidiae). These genera included *Aureobasidium, Candida, Cryptococcus, Metschnikowia, Pseudozyma, Rhodotorula, Sporobolomyces* and *Tilletiopsis* (Molnár et al. 2008). The insects were sampled from two different sites and the yeast genera found at the two sites differed notably. This may be an indication of the transient association between yeasts and these insects. Moreover, it indicated that the gut yeast community of a single insect species may differ between geographically separated areas.

Most known yeast-beetle interactions involve beetles (Coleoptera) that live on plants. The close association between beetles and different plants (especially with angiosperms) may present a major cause for beetle diversity (Ganter 2006) with ca. 350 000 described species (Lachance 2006). Whether the yeasts associated with beetles follow the same trend, is unknown. To predict the number and diversity of yeasts that may be associated with beetles, it needs to be determined what proportion of beetles harbour yeasts. Interactions of yeasts with phytophagous beetles (Chrysomelidae) are uncommon compared to tree boring species and floricolous nitidulids (Lachance 2006). However, studies on yeasts associated with leaf beetles are lacking.

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Chapter 2

Symbiotic interactions of culturable microorganisms with the nickel hyperaccumulator *Berkheya coddii* and the herbivorous insect *Chrysolina clathrata*

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2.1 Introduction

The weathering of ultramafic rocks, which consist of ca. 70% ferromagnesian or mafic minerals, creates ultramafic (serpentine) soils containing elevated concentrations of heavy metals (HMs) such as nickel (Ni) (Brady et al. 2005). Serpentine ecosystems have long fascinated scientists because of the unique flora they support, the adaptive challenges they pose to plants, and the difficulties they create for agriculture and more recently, for ecological restoration (Harrison and Rajakaruna 2011). Also, serpentine flora has been treated as a model system for understanding mechanisms of adaptation, ecotypic differentiation, and the linkage between natural selection and speciation (Harrison and Rajakaruna 2011). The vast majority of these studies focussed on the ecology and evolution of serpentine plant species (Branco and Ree 2010). Comparatively few studies have been conducted to describe the microbiota of serpentine ecosystems, despite the contention that microorganisms play an important role in the adaptation of plants and animals in these environments (Brady et al. 2005).

Berkheya coddii Roessler (Asteraceae) is a Ni hyperaccumulating plant endemic to the ultramafic soils of the Barberton area in the Mpumalanga Province, South Africa. Initially, research on this plant focused on its application as a phytomining and phytoremediation agent and it was determined that *B. coddii* is superior to other hyperaccumulators in both biomass production and Ni accumulation ability (Robinson et al. 1997; Salt et al. 1998; Brooks et al. 1998; Brooks and Robinson 1998; Brooks et al. 2001; Harris et al. 2009; Robinson et al. 1999). During the past decade the ecophysiology of this plant, its herbivorous grazers and their predators have been studied intensively (Augustyniak et al. 2002, 2007; Mesjasz-Przybyłowicz et al. 2001, 2002; Turnau and Mesjasz-Przybyłowicz 2003; Migula et al. 2007, 2011; Moradi et al. 2010; Robinson et al. 2003). The metals hyperaccumulated by plants are generally toxic in low doses and the metal levels in these plants may render them relatively toxic to other organisms with which they interact (Boyd et al. 1994). Dried leaves of *B. coddii* contain up to 7.6 % Ni (Mesjasz-Przybyłowicz et al. 2004) but the Chrysomelid beetle *Chrysolina clathrata* Clark (formerly *Chrysolina pardalina*) can develop fully and proliferate through several generations feeding exclusively on *B. coddii* leaves. This beetle, first reported in 1999 (Mesjasz-Przybyłowicz and Przybyłowicz 1999, 2001), has an extremely low bioaccumulation factor attributed to adaptive physiological mechanisms, particularly of the

Malphigian tubules and the midgut. Malphigian tubules remove Ni from the haemolymph, and interestingly, the adaptive mechanisms of the midgut seem to involve a high cell turnover rate. Nickel is concentrated in the vacuolar space of these cells, and is excreted along with the faeces (Nakonieczny 2007).

Uptake and sequestration of toxic materials represent an interesting biological puzzle and the impact of high Ni concentrations on the various trophic levels of this particular food web has been extensively studied. The involvement of microorganisms in the ecophysiological structure, based on ultramafic soil, is a major source of interest. Pathogenic fungal associations with hyperaccumulators were the first to be investigated in serpentine environments due to the impact these fungi may have on phytomining efforts. Boyd and Martens (1992) hypothesized that the accumulation of HMs in aerial tissues may function as a self-defence strategy evolved in hyperaccumulating plants against some natural enemies, such as herbivores and microbial pathogens. It was subsequently demonstrated that elevated Ni levels in hyperaccumulating plants may act as protection against phytopathogens (Boyd et al. 1994). Although some tests led to contradictory results, this elemental defence hypothesis is gaining much supporting evidence (Rascio and Navari-Izzo 2011).

High levels of HMs are almost universally toxic, affecting fungi directly through disruption of cellular integrity and enzymatic inhibition, and indirectly through the production of free radicals (Branco and Ree 2010). However, the presence of mycorrhizal fungi has been shown to be compulsory for *B. coddii*'s survival, increasing both the biomass and Ni contents of the plant's shoots. Moreover, mycorrhizae have a significant impact on the availability and distribution of HMs in the roots of *B. coddii* (Turnau and Mesjasz-Przybyłowicz 2003; Orłowska et al. 2008; Orłowska et al. 2011). Also, efforts in finding novel HM biosorbents led to the discovery of HM resistant fungal and bacterial endophytes of HM hyperaccumulating plants (Luo et al. 2011; Xiao et al. 2010). Certain endophytic organisms can enhance plant growth, increase plant resistance to pathogens, HMs, drought and even herbivores (Rajkumar et al. 2009). Endophytes of hyperaccumulators can absorb specific HMs due to the fact that they are exposed to high concentrations of a certain heavy metal hyperaccumulated by their host (Xiao et al. 2010). Studies have also shown that microorganisms can adapt to HMs after prolonged exposure (Ezzouhri et al. 2009; Congeevaram et al. 2007; Ahmad et al. 2005). This implies

that similar to endophytic microorganisms, pathogens can also adapt to high HM concentrations and therefore circumvent a plant's HM hyperaccumulating defense strategy (Boyd et al. 1994).

Herbivores of hyperaccumulators and their microbial symbionts face the same challenges, regarding Ni toxicity, as those microorganisms associated with the hyperaccumulators. Plant feeding insects were often found to be in symbiotic relationships with yeasts (Suh et al. 2008). In some cases, yeast clades or metabolic guilds were found to have specific associations with certain insects. Ascomycetous yeasts were detected in the insect gut, sometimes as endosymbionts in special compartments (Gullan and Cranston 2010). These unicellular fungi detoxify plant metabolites or provide enzymes to attack plant cell walls that are intractable to insects. Also, many insects rely on microbial enzymes for the fermentation of food resources to improve the nutritional content thereof (Gullan and Cranston 2010). It was stated that these microbial interactions may allow insects to occupy habitats with relatively little genetic expense (Gullan and Cranston 2010). However, over the years direct relationships between fungi and insect evolution generally remained poorly studied (Suh et al. 2004) and the yeasts that may be associated with *C. clathrata* are no exception.

To obtain a better understanding of potential symbiotic associations between *B. coddii*, its herbivore *C. clathrata,* and Ni tolerant microorganisms, the aim of the current study was twofold: (a) to identify culturable microbiota that may associate with the leaves of *B. coddii* plants and (b) to uncover how these organisms may interact not only with the plant but also with the second trophic level. For this purpose, we focused on yeasts as a microbial intertrophic link between *B. coddii* and *C. clathrata* since no such interactions have been documented.

2.2 Materials and Methods

2.2.1 Experimental design

In this study we used *Berkheya coddii* plants, originally sampled at the Agnes Mine, Groenvaly and the Songimvelo Game Reserve (Barberton area, Mpumalanga Province, NE South Africa), as described in a previous paper (Augustyniak et al. 2002). Eighteen of these

plants were each replanted in 17 L plastic potting bags containing a mixture of serpentine soil, collected from the sampling sites. The soil mixture was fertilized with a 1: 5 ratio of compost and ± 50 g of slow release phosphorus. In addition, the soil of each plant was spiked every week with 100 ml of a 100 ppm Ni solution prepared from reagent grade $NiCl{\cdot}(6H_2O)$ (Merck). *Chrysolina clathrata* beetles, sampled from Groenvaly (Augustyniak et al. 2002), were reared in Petri dishes at room temperature (25°C). They were fed young leaves picked from the cultured plants. A natural photoperiodic regime of 12 h light, 12 h dark was followed and humidity was maintained with moist tissue paper. However, controlling the humidity in the Petri dishes was not possible due to the destabilizing effect of regularly adding fresh leaves.

2.2.2 Colorimetric determination of Ni concentrations in organic material

We used a colorimetric method to confirm that our laboratory reared *B. coddii* plants hyperaccumulated Ni and that the beetles sequestrated the metal in their faeces. Oven dried (70-80 °C) finely cut leaves and faeces were weighed before being wet digested in 250 ml conical flasks containing 15 ml of a 1:1 concentrated perchloric-nitric acid mixture on a hot plate. The digestion was continued until the contents of the flask turned colourless and the volume of the digest was reduced to between 2 and 3 ml. After cooling, the content of each flask was transferred to a 25 ml volumetric flask. Each conical flask was washed three times with 5 ml distilled water and the resulting solutions were transferred to the volumetric flask after which the volume was adjusted with distilled water (Wahid et al. 1985).

The Ni concentration in the digest solution was subsequently determined with the dimethylglyoxime (DMG) colorimetric method adapted from Kentner and co-workers (1969). A 5 ml aliquot of the digest solution was adjusted to pH 9-10 with a few drops of concentrated ammonium hydroxide (NH_4OH) (Merck). To remove any interfering iron ions, 2.5 ml of a 20 % (w/v) aqueous sodium citrate ($Na_3C_6H_3O_7$) solution was added. Nickel ions were precipitated using 2.5 ml of a fresh 1 % (w/v) DMG (Sigma) in 95 % ethanol solution. The Ni-DMG complex was extracted once into 10 ml of chloroform using a separatory funnel. Two more extractions into 5 ml portions of chloroform were performed and all the extracts (ca. 20 ml) were collected in glass beakers. The Ni ions were back extracted into three 5 ml portions of 1 M hydrochloric acid (HCl); shaking each extraction for 30 seconds. To ensure complete oxidation of Ni(II) to Ni(IV), 1 ml of saturated bromine water was added to the hydrochloric

acid extract solution and left for 15 minutes. Subsequently the pH of the solution was adjusted to 10.4 ± 0.05 with concentrated NH_4OH and the Ni(IV) complex was developed with 1 ml of the DMG reagent. The solution was transferred to a 50 ml volumetric flask and the volume was made up with distilled water. The absorbance was read at 442 nm (Biorad Smartspec Plus spectrophotometer), using quartz cuvettes with a 1 cm path length, against a blank carried through the same procedure. Triplicate absorbance values were used to calculate the Ni concentration from a calibration curve prepared with reagent grade nickelchloride hexahydrate ($NiCl{\cdot}6H_2O$) (Merck) salt solutions. The Ni concentration per gram of dry leaves or faeces was determined.

2.3 Isolation of culturable microorganisms

2.3.1 Filamentous fungi from leaves

Twelve leaves were aseptically sampled from each of four different healthy plants and transported in sterile 1 L glass jars and processed immediately upon arrival to the laboratory. Some leaves had black or grey spots indicating possible infection. The leaves were aseptically cut into smaller pieces (ca. 1 cm^2) which were used to inoculate potato dextrose agar (PDA; Biolab, Merck, Wadeville, Gauteng, South Africa) and malt extract agar (MEA; Biolab, Merck, Wadeville, Gauteng, South Africa) plates. Filamentous fungal growth was observed after 1 to 4 days of incubation at 26 °C and subsequently 43 randomly selected colonies were isolated and purified by repetitive culturing. Based on colony and microscopic morphology, 10 different isolates were selected for molecular identification.

The 10 filamentous fungal isolates were subsequently tested for cellulase production by growing them on carboxymethylcellulose plates (CMC) (0.67 % Yeast Nitrogen Base (Becton, Dickinson and Company Sparks, MD 21152, USA), 0.5 % Carboxymethyl-Cellulose (Sigma-Aldrich, GmbH, Steinheim, Germany), 2 % Bacteriological Agar). The plates were inoculated with 5 mm fungal plugs, which were isolated with the back end of a sterilized Pasteur pipette from one week old colonies cultured on MEA plates. After seven days of incubating the inoculated CMC plates at 26 °C, the plates were stained for 10 minutes using 0.1 % Congo Red (B and M Scientific, Cape Town, South Africa) followed by destaining for 5 minutes with 1

M NaCl (KIMIX, Cape Town, South Africa). Cellulase activity was indicated by clear zones surrounding the fungal colonies on the stained CMC plates.

2.3.2 Yeasts from leaves and faeces

Yeasts were isolated and enumerated from both the leaves and beetle faeces. Young leaves, sampled from four plants, were aseptically cut into smaller pieces (ca. < 1 cm^2) and 10 g (wet weight) of these leaf pieces was used to prepare a dilution series with physiological saline solution. Fresh faeces less than two days old were scraped from the Petri dishes in which the insects were reared. Subsequently 0.1 g (wet weight) pooled samples of the faeces were serially diluted. The dilutions were plated onto a yeast selective medium consisting of yeast malt extract agar (YM) containing 0.2 % chloramphenicol (Sigma) and 1 % biphenyl (Sigma), as well as onto the same medium but amended with 1 mM Ni (YMN). Plates were incubated at 26 °C for at least 48 hours after which the colony forming units (CFUs) per gram of leaves or faeces was calculated. The isolation and enumeration experiments were performed in triplicate on four separate sampling occasions over a one month period. The difference between the means of the number of yeasts occurring on the leaves and in the faeces was determined using the t-Test for two samples with unequal variances ($p<0.01$). Data analysis was performed using Microsoft Excel 2003 software (Microsoft Corporation). Randomly selected yeast colonies isolated from both the YM and YMN plates were repetitively cultured to obtain pure cultures. Yeast species were first classified according to colony morphology and restriction fragment length polymorphism (RFLP) analysis, and then the species were identified using the molecular techniques described below.

2.3.3 Endophytic bacteria

Leaves, sampled as above, were surface sterilized with an immersion sequence treatment adapted from Reissinger and co-workers (2001). First, *B. coddii* leaves were submerged in 95% ethanol for one minute, secondly in 10 % (v/v) peracetic acid (Peratize, Medichem, Atlantis, Western Cape, South Africa) for 5 minutes and thirdly again in 95 % ethanol for 30 seconds. Finally, the leaves were rinsed in sterile distilled water and 1 ml of this water was plated out on nutrient agar (NA; Biolab, Merck, Wadeville, Gauteng, South Africa) plates to test for sterility. Sections (ca. 1 cm^2) of the surface sterilized leaves were used to inoculate NA plates. Bacterial colonies, originating from leaf sections, were randomly selected and

streaked out on NA plates. Based on morphology, six different isolates were selected for molecular identification.

2.4 Molecular classification and identification of isolates

2.4.1 DNA extraction

Genomic DNA was extracted from selected bacterial and filamentous fungal isolates using the ZR Fungal/Bacterial DNA Kit™ (Cat. Nr: D6005, Zymo Research Corp., USA). A manual method described by Vreulink et al. (2010) was used to isolate genomic DNA from the yeast isolates.

2.4.2 RFLP analysis of yeast isolates

Prior to phylogenetic identification, the internal transcribed spacer (ITS) region of the ribosomal gene cluster (ITS1/5.8S/ITS2) of yeast isolates was amplified using PCR with the primer set ITS1 and ITS 4 (Table 2.1) (White et al. 1990, Javier et al. 2003; Esteve-Zarzoso et al. 1999; Guillamón et al. 1998). The 50 µl PCR mixture contained 25 µl of master mix (2x) (Fermentas International Inc., Burlington, Ontario, Canada), 2 µl of each primer (10 µmol/L) (Inqaba biotech Industries, Pretoria, South Africa) and 2 µl template DNA. Amplification was performed in a Perkin-Elmer 2400 thermal cycler at an initial denaturation of 95 °C for 3 min, 36 cycles consisting of denaturation at 95 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min, as well as a final extension at 72 °C for 7 min.

The amplified ITS region was digested with the restriction endonucleases *Hin*6I, *Hin*fI and *Mbo*II according to the manufacturer's specifications (Fermentas). The resulting fragments were separated on a 2 % (w/v) agarose gel containing ethidium bromide and photographed (Gene Flash, Syngene Bio Imaging, Cambridge, UK). Banding patterns, as well as sizes of the fragments were compared to a 100 bp DNA Ladder (GeneRuler, Fermentas).

2.4.3 PCR, sequence identification and sequence similarity

The taxonomic informative gene loci of selected isolates, representative of each morphological and RFLP group, were amplified using PCR with the primer sets listed in Table 2.1. The PCR products were purified by means of a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals (Pty) Ltd., S.A.) and sequenced using a Hitachi 3730xl DNA

Analyzer (Applied Biosystems, Foster City, California, USA). The resulting sequences were adjusted using Chromas Lite version 2.01 (Technelysium Pty. Ltd.). A search for highly similar sequences in GenBank was performed using the Basic online Local Alignment Search Tool (BLAST) on the NCBI website. Sequences with ≥ 99 % homology to sequences obtained from our isolates were selected for further analysis. Additionally, the relevant sequences of type strains stored in the Centraalbureau voor Schimmelcultures (CBS; filamentous fungi and yeasts), and NITE Biological Resource Center (NBRC, bacteria) culture collections, were obtained from GenBank.

Sequences were aligned using the online multiple alignment program for amino acid or nucleotide sequences (MAFFT version 6, Computational Biology Research Center (CBRC) National Institute of Advanced Industrial Science and Technology (AIST) , Japan) using the L-INS-i algorithm. The resulting alignments were edited and re-aligned manually where necessary using the BioEdit Sequence alignment editor (version 7.0.9.0). Subsequently, the alignments were imported into Mega V.5.05 and neighbour joining trees were constructed. The quality of the branching patterns was assessed by bootstrap resampling of the data sets with 1000 replications. Sequences obtained in this study were submitted to GenBank.

Table 2.1: Taxonomically informative gene loci (TIGL) and primers used to identify microbial isolates.

Isolates	TIGL	Primers	Primer sequence
Filamentous fungi	ITS1/5.8S/ITS2	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'
		ITS4	5'-TCCTCCGCTTATTGATATGC-3'
Yeasts	D1/D2 (26S rDNA)	F63	5′-GCATATCAATAAGCGGAGGAAAAG-3′
		LR3	5′-GGTCCGTGTTTCAAGACGG-3′
Bacteria	16S rRNA	FWD	5'-AGTTTGATCCTGGCTCAG-3'
		REV	5'-TACCTTGTTACGACTTCACCCCA-3

2.4.4 Minimum inhibitory Ni concentration

The resistance to Ni(II) was estimated using a dilution method that was adapted from Ezzouhri et al. (2009), which entails determining the minimum inhibitory concentration (MIC) of Ni for each microbial isolate. Nickel ions were added to microbiological growth media at various concentrations as listed in Table 2.2.

Table 2.2: Microbiological media and Ni concentrations used to determine the Ni MIC of isolated microbes.

Isolates	Media	[Ni] range (mM)
Filamentous fungi	PDA	0;1; 2.5; 5; 7.5; 10; 12.5; 15
Yeasts	YM	0; 0.5; 1; 2.5; 5; 7.5
Bacteria	LB	0; 1; 2.5; 5; 7.5; 10

PDA= Potato dextrose agar; YM= Yeast Malt extract broth; LB= Luria Bertani broth

Potato dextrose agar plates were inoculated with 5 mm plugs of young filamentous fungal colonies cultured on PDA. The Ni MIC tests of the yeast and bacterial isolates were performed with test tubes containing 10 ml YM and LB (Luria-Bertani) broth respectively. Ten microlitres of a starter culture, grown to stationary phase in either YM or LB, were used to inoculate each of the test tubes. The inoculated plates and test tubes were incubated at 26 °C for at least 7 days. The Ni MIC of each isolate was identified as the lowest Ni concentration that visibly inhibited growth.

2.5 Results

2.5.1 Ni concentration in leaves and faeces

In our study the Ni concentration of the leaves from three cultured *B. coddii* plants, were above the definitive hyperaccumulator concentration of >1000 µg/g dry weight of leaves (Brooks et al. 1998) with an average of 2700 ± 1750 µg/g Ni. The Ni concentration levels in the beetle's faeces were almost 10 times higher (24 850 ± 2680 µg/g) than that found in the leaves.

2.5.2 Filamentous fungal isolates

Our study yielded 9 filamentous fungal species (Figure 2.1) namely *Alternaria alternata* (JX310562; JX310567), *Alternaria infectoria* (JX310564), *Aspergillus nomius* (JX310565), *Bipolaris australiensis* (JX310570), *Bipolaris cynodontis* (JX310561), *Cladosporium tenuissimum* (JX310566), *Epicoccum nigrum* (JX310563), *Fusarium oxysporum* (JX310569) and *Penicillium olsonii* (JX310568).

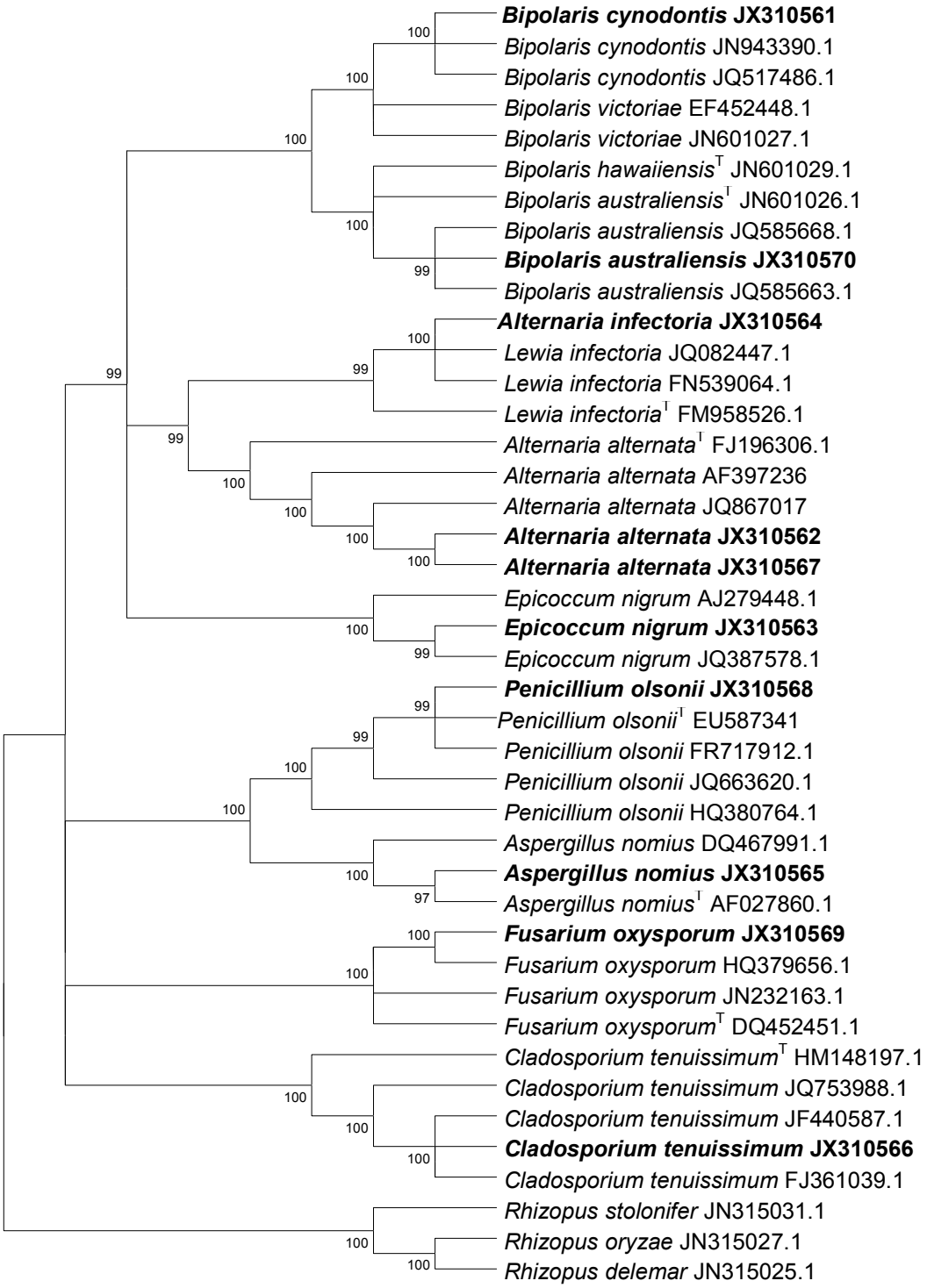

Figure 2.1: Neighbour joining tree comparing sequences of filamentous fungi isolated in the present study (names in bold) to sequences from GenBank (with accession numbers). The tree was constructed using Mega V. 5.05 with confidently aligned ITS1/5.8S/ITS2 rDNA sequences. T = Genbank sequences of type strain cultures stored in the Centraalbureau voor Schimmelcultures (CBS) culture collection. Bootstrap analysis was performed with 1000 repetitions and bootstrap values are shown next to branches. The cut-off value was set at 95. Three species of *Rhizopus* were selected as outgroup.

Most of our isolates showed cellulase activity, which may be a potential plant virulence factor (Temuujin et al. 2011) while all the isolates showed resistance to Ni (Table 2.3). *Aspergillus nomius* and *P. olsonii* showed very high resistance to Ni with an MIC of 10 mM which may be an indication of a close, perhaps pathogenic, association with *B. coddii.* Both representatives of *Alternaria* isolated during this study showed high resistance to Ni with an MIC of 5 and 7.5 mM for *A. alternaria* and *A. infectoria*, respectively. Both representatives of the genus *Bipolaris* had a Ni MIC of 5 mM (Table 2.3). The *C. tenuissimum* and *E. nigrum* strains showed relatively low Ni resistance compared with the other filamentous fungal isolates with a MIC of 2.5 mM (Table 2.3).

Table 2.3: Filamentous fungal species isolated from *B. coddii* leaves and their minimum inhibitory Ni concentration (MIC). The ability of isolates to produce cellulose was determined using carboxymethyl cellulose agar plates.

Species	Ni MIC (mM)	Cellulase	Plant assocation‡
Alternaria alternata	5.0	-	Saprophyte/Pathogen
Alternaria infectoria	7.5	+	Saprophyte/Pathogen
Aspergillus nomius	10.0	+	Saprophyte
Bipolaris australiensis	5.0	+	Pathogen
Bipolaris cynodontis	5.0	+	Pathogen
Cladosporium tenuissimum	2.5	+	Pathogen
Epicoccum nigrum	2.5	+	Saprophyte
Fusarium oxysporum	5.0	+	Pathogen
Penicillium olsonii	10.0	+	Pathogen

‡ Based on recent reports where these fungi were isolated from different plant species/material

2.5.3 Yeasts isolated from phylloplane and beetle's faeces

Using dilution plates prepared with YM agar a significant ($p<0.01$) difference was found between the total number (log CFU/g) of yeasts on the phylloplane (0.94 ± 0.72; n=12) and in the beetle's faeces (7.6 ± 0.43; n=12). No yeasts were detected on the leaves when YMN agar was used as enumeration medium. Interestingly, dilution plates prepared with YM and YMN yielded the same amount of CFUs originating from the beetle's faeces.

Restriction fragment length polymorphism (RFLP) analysis of 113 randomly selected yeast isolates, originating from the phylloplane of *B. coddii* and the faeces of *C. clathrata,* yielded eight banding pattern groups (Table 2.4), which correlated with distinct yeast species (Figure 2.2). The leaves of *B. coddii* yielded five species represented by 13 strains (Table 2.4).

We isolated representatives of three *Cryptococcus* species i.e. *Cryptococcus albidus, Cryptococcus flavescens,* and *Cryptococcus oeirensis,* as well as *Rhodotorula mucilaginosa* from the leaves, while only one ascomycetous species, *Meyerozyma guilliermondii* occurred in this habitat (Table 2.4). The Ni resistance varied both inter- and intraspecifically. With a Ni MIC of 0.5 mM representatives of *C. oeirensis* showed the least resistance among the isolates, representatives of *C. albidus* and *R. mucilaginose* were more resistant (Ni MIC 1 mM), while isolates representing *C. flavescens* and *M. guilliermondii* were the most resistant (Ni MIC 2.5 mM).

Three yeast species were detected in faeces of the adult beetles (Table 2.4). Strains representing *M. guilliermondii* and *C. intermedia* were isolated from the faeces on three out of the four sampling occasions. Also, representatives of these two species were the most abundant of the randomly selected isolates (Table 2.4). Only two *C. flavescens* strains could be isolated from four sampling occasions. The Ni resistance of these yeast strains ranged from 2.5 mM (*C. intermedia* and *C. flavescens*) to 5 mM (*M. guilliermondii*). None of the yeasts species detected in the adult beetle's faeces were found in the larval faeces, which yielded two ascomycetous species, *Debaryomyces hansenii* and *Wickerhamomyces anomalus.* Compared to yeasts strains isolated from the faeces of the adult beetles (Table 2.4), representatives of these two species showed low resistance towards Ni with a MIC of 1.0 and 0.5 mM, respectively.

Table 2.4: Yeast species isolated from leaves of laboratory reared *Berkheya coddii* plants and *Chrysolina clathrata* larvae and adult beetle faeces. Collectively, we isolated 113 yeast strains and used RFLP analysis to distinguish different species based on DNA banding patterns. The identities of strains representative of each RFLP banding pattern group were determined by comparing the 26S rRNA sequences of our isolates to highly similar sequences found on GenBank using BLAST and phylogenetic analysis (fig. 2). Also, the minimum inhibitory Ni concentration (MIC) was determined of strains representing each species.

Species	Source	No. of Isolates (n^a)	Ni MIC (n^b)	Restriction fragments		
				HinfI	Hin6I	BsuRI
Candida intermedia	AF	30 (90)	2.5 (2)	320, 210, 185	210, 185	325, 180
Cryptococcus albidus	BL	6 (13)	1.0 (2)	310, 240	290, 270	245, 195, 180
Cryptococcus flavescens	AF	2 (90)	2.5 (2)	290, 175	250, 210, 175	270, 210, 170
Cryptococcus flavescens	BL	1 (13)	2.5 (1)	290, 175	250, 210, 175	270, 210, 170
Cryptococcus oeirensus	BL	2 (13)	0.5 (2)	-	-	-
Debaryomyces hansenii	LF	2 (10)	1.0 (2)	305	290, 270	200, 165
Meyerozyma guilliermondii	AF	58 (90)	5.0 (5)	270, 240	250, 210	205, 180, 140, 115, 90
Meyerozyma guilliermondii	BL	1 (13)	2.5 (1)	270, 240	250, 210	205, 180, 140, 115, 90
Rhodotorula mucilaginosa	BL	3 (13)	1.0 (2)	330, 200	275, 200	195
Wickerhamomyces anomalus	LF	8 (10)	0.5 (4)	290	590, 340	660, 590, 290, 230

AF= Adult beetle faeces; LF= Larvae faeces; BL= *B. coddii* leaves; (n^a) = total number of isolates from each source; (n^b) = number of strains tested.

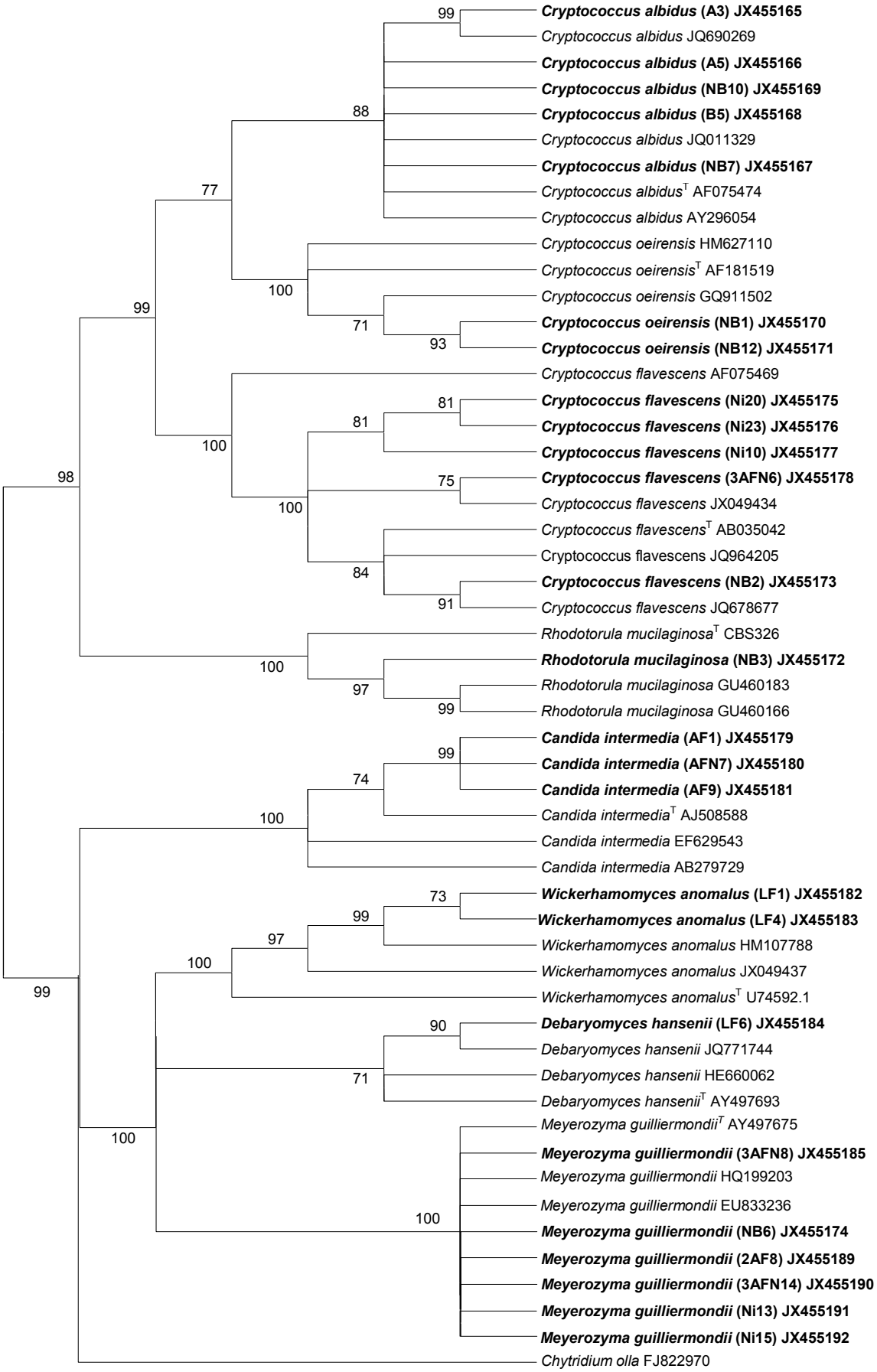

Figure 2.2: Neighbour joining tree comparing sequences of Yeast strains isolated in the present study (names in bold) to sequences from GenBank (with accession numbers). The tree was constructed using Mega V. 5.05 with confidently aligned 26S rDNA sequences. T = Genbank sequences of type strain cultures stored in the Centraalbureau voor Schimmelcultures (CBS) culture collection. Bootstrap analysis was performed with 1000 repetitions and bootstrap values are shown next to branches. The cut-off value was set at 70. *Chytridium olla* was selected as outgroup.

2.5.4 Endophytic bacteria

In this study, we were able to isolate four bacterial species which belong to the genus *Bacillus* and one *Lysinibacillus* sp. (Fig. 2.3; Table 2.5). All the isolates were resistant to Ni. However there was interspecific variation on the degree of resistance. The representatives of *Bacillus cereus* and *Bacillus megaterium* had higher Ni resistance (Ni MIC of 5 mM) than the representatives of *Bacillus nealsonii, Bacillus subtilis* and *Lysinibacillus fusiformis* (formerly *Bacillus fusiformis*).

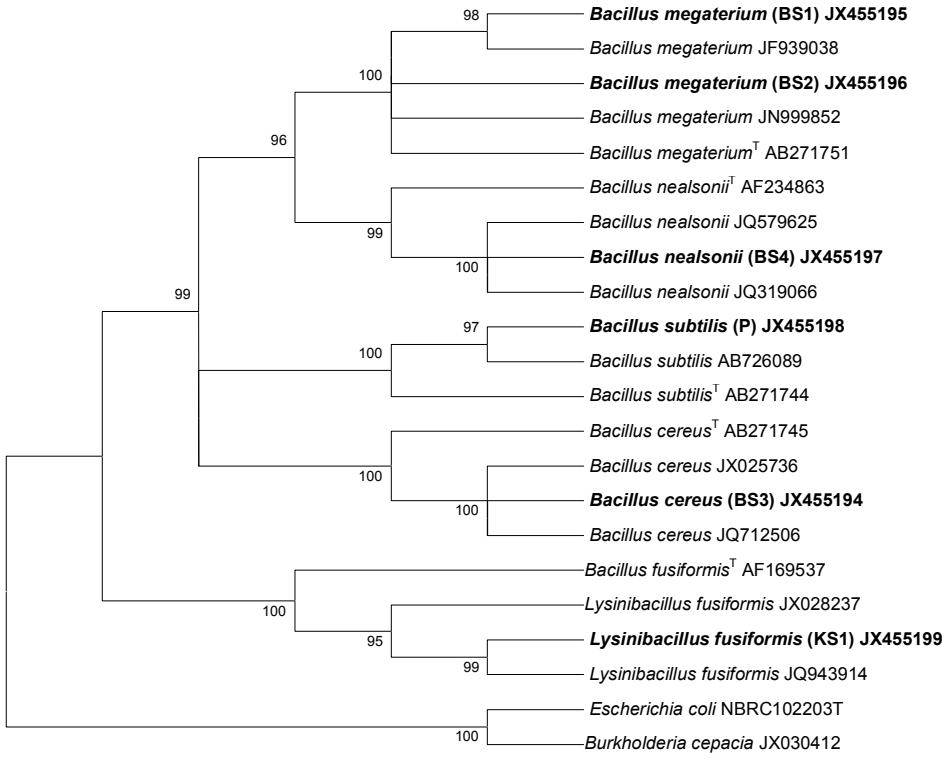

Figure 2.3: Neighbour joining tree comparing sequences of endophytic bacterial strains isolated in the current study (names in bold) to sequences from GenBank (with accession numbers). The tree was constructed using Mega V. 5.05 with confidently aligned 16S rDNA sequences. T = Genbank sequences of type strain cultures stored in the NITE Biological Resource Center (NBRC) culture collection. Bootstrap analysis was performed with 1000 repetitions and bootstrap values are shown next to branches. The cut-off value was set at 70. Strains of *Escherichia coli* and *Burkholderia cepacia* were selected as outgroups.

Table 2.5: Endophytic bacteria isolated from surface sterilized leaves of laboratory reared *Berkheya coddii*. The identities of the isolates were determined by comparing the 16S rDNA sequences of our isolates to highly similar sequences found on GenBank using BLAST and phylogenetic analysis (Fig. 2.3). Also, the minimum inhibitory Ni concentration (MIC) was determined for each species.

Species	No. of Isolates	Ni MIC
Bacillus cereus	1	5.0
Bacillus megaterium	2	5.0
Bacillus nealsonii	1	2.5
Bacillus subtilis	1	2.5
Lysinibacillus fusiformis	1	2.5

2.6 Discussion

2.6.1 Ni concentration in leaves and faeces

The Ni concentration in the leaves (ca. 3000 µg/g) and faeces (ca. 25 000 µg/g) of our laboratory reared plants and insects were comparable to those of wild specimens. The Ni concentration of the leaves of *Berkheya coddii* plants growing in the serpentine soil of the Barberton Greenstone Belt varies from 4 900 to 76 000 µg/g (Augustyniak et al. 2002, Mesjasz-Przybyłowicz et al. 2004). Previously, the average concentration of Ni in *C. clathrata* faeces was found to be 35 000 µg/g (Mesjasz-Przybyłowicz et al. 2002).

2.6.2 Filamentous fungal isolates

The filamentous fungal genera encountered by us in the current study (Table 2.3) are similar to those isolated previously from another Ni hyperaccumulator endemic to South Africa, *Senecio coronatus* (Thunb.) Harv. (Asteraceae) (Mesjasz-Przybyłowicz and Przybyłowicz 2001). In the latter case, isolates represented the genera *Alternaria* Nees. (Pleosporaceae) *Aspergillus* Link (Trichocomaceae) and *Sphaerotheca* Fusca (Fr.) Blumer (Erysiphaceae).

The high Ni resistance of our filamentous fungal isolates from *B. coddii* leaves (Table 2.3) is noteworthy since Ni has been used commercially as a systemic fungicide and it is hypothesized that the hyperaccumulating strategy is used as a defense mechanism against insect herbivores and pathogens (Boyd and Martens 1992; Boyd et al. 1994). It is therefore

possible that filamentous fungi encountered in our study may have adapted to high Ni concentrations after continuous exposure to this HM (Vadkertiová and Sláviková 2006). Furthermore our results are consistent with findings by Ezzouhri and co-workers (2009) and Saxena and co-workers (2006) who isolated highly HM resistant *Aspergillus, Penicillium* and *Fusarium* species from aqueous HM contaminated environments.

The filamentous fungal species encountered by us (Table 2.3) *on B. coddii* leaves were previously isolated by others from a wide diversity of plant associated habitats. *Aspergillus nomius,* known for its ability to produce aflatoxins, is widely distributed and has been isolated from tree nuts, sugar cane, an assortment of seeds and grains as well as insects (Horn and Moore 2011). Vega and co-workers (2006) previously isolated *P. olsonii* from coffee plants in a survey for endophytic fungi.

Alternaria alternata and *A. infectoria* are cosmopolitan imperfect fungi that are generally saprophytic (Hatta et al. 2002). Seven pathogenic variants (pathotypes) however are known, which produce necrotrophic host-specific toxins and cause disease in a variety of plants (Hatta et al. 2002). Species in the genus *Cladosporium* are often encountered on the phylloplane in temperate regions (O'Donnell and Dickinson 1980). Together with *Alternaria* species, they form a major component of both the phylloplane and litter decomposing communities and are well adapted for survival, growth and dispersal on aerial plant surfaces (O'Donnell and Dickinson 1980). *Cladosporium tenuissimum* is known as a hyperparasite of rust fungi (Nasini et al. 2004).

The genus *Cochliobolus* (anamorphs *Bipolaris*, *Curvularia*) comprises many destructive necrotrophic plant pathogens such as *B. sorghicola, B. zeicola* (Winder and Van Dyke 1990) and *B. sorokiniana* (Bhatti and Bhutta 2002) that cause severe crop losses worldwide (Manamgoda et al. 2011). *Cochliobolus australiensis* is an opportunistic human pathogen but was also isolated from banana foliage in South Africa (Manamgoda et al. 2011). *Bipolaris cynodontis* produces a selective phytotoxin, bipolaroxin, previously considered as potential herbicide (Sugawara et al. 1985).

Epicoccum nigrum is ubiquitous, colonizing different types of soils and host plants (Faváro et al. 2011). This species is considered saprophytic but can display an endophytic lifestyle. Also, it has been used as a biological control agent against fungal pathogens of economically important crops (Faváro et al. 2011). The low Ni resistance of these two isolates and the antagonistic effects of other strains of these species may be an indication that our strains do not interact with the living leaf tissue of *B. coddii.* These species may be transient inhabitants of the leaf surface and will probably be more active as saprophytes degrading dead plant material.

The genus *Fusarium* contains notorious plant pathogenic fungi with a wide variety of infection strategies and hosts (Michielse and Rep 2009). Certain strains of *Fusarium oxysporum* (Table 2.3) may invade roots, causing wilt disease through colonization of xylem tissue and displays apparent gene-for-gene relationships with several hosts (Michielse and Rep 2009). However, most *F. oxysporum* strains are not pathogenic and survive saprophytically in the soil or endophytically inside the plant tissue (Kidd et al. 2011).

Although strains of all the above mentioned fungal species have been described as pathogens, saprophytes, epiphytes or endophytes, the delineation between these categories may be only temporal or circumstantial. Fungal endophytes are defined as fungi that colonize plants without causing visible disease symptoms. However, Schulz and Boyle (2005) hypothesized that there are no neutral endophyte-host interactions but rather a balance of antagonisms. There is always at least a degree of virulence on the part of the fungus enabling infection, whereas defence of the plant host limits development of fungal invaders and disease (Schulz and Boyle 2005). The developmental stage and physiological state of a plant can determine if an endophytic fungus can grow asymptomatically or cause disease. Also, weakened plants are exploited by opportunistic pathogens. The Ni concentration in *B. coddii*'s tissues may influence the lifestyle of fungi associated with the plant. Whether the Ni resistance of the fungal strains isolated in this study is an indication of virulence, should be further investigated.

2.6.3 Yeasts isolated from phylloplane and beetle's faeces

The dominant yeast species on the phylloplane of *B. coddii* were found to be basidiomycetes (Table 2.4). These results support the contention that basidiomycetous yeast species belonging to the genera *Cryptococcus* and *Rhodotorula,* are usually dominant yeast species among phylloplane communities (Fonseca and Inácio 2006). In contrast, the ascomycetous yeast species *Meyerozyma guilliermondii* and *C. intermedia,* appeared to be the dominant yeasts in the adult beetle's faeces, especially since the only other species found in the faeces, *C. flavescens,* was represented by only two out of 90 isolates. This indicated that unlike representatives of *M. guilliermondii* and *C. intermedia,* strains of *C. flavescens* do not seem to proliferate during passage through the beetle's gut.

Isolates representing *C. flavescens* and *C. intermedia* showed the same Ni MIC (2.5 mM), which was lower than the Ni MIC (5 mM) of *M. guilliermondii* obtained from adult beetle faeces (Table 2.4). Interestingly, while Ni resistance of *C. flavescens* strains from the leaves and faeces were the same (Ni MIC 2.5 mM), the *M. guilliermondii* strains from the faeces had a higher Ni resistance than the strain from the phylloplane representing this species. It therefore seems that the *M. guilliermondii* strain isolated from the faeces has adapted to the high Ni levels by being able to be more resistant to higher levels of the HM, compared to representatives of this yeast species on the phylloplane.

The difference in yeast species obtained from the adult and larvae beetle faeces may indicate that a shift in yeast populations in the faeces occur during development of the beetle. Although more surveys are needed to confirm the existence of such a shift, it was previously proposed that population shifts may occur in the microbial community of a host's gut when its different developmental stages are compared (Nicholson et al. 2012). Thus, it is tempting to speculate that the ability of *C. clathrata* to sequester Ni during its development into an adult beetle may be an important determinant of the yeast community composition in its gut.

It is notable that two of the three yeast species isolated from the faeces of the adult beetles were also found on the leaves i.e. *C. flavescens* and *M. guilliermondii.* This indicates horizontal transfer of yeasts from the phylloplane to the beetle which may influence the gut microbial community of the beetle. The apparent absence of *C. intermedia, D. hansennii* and

W. anomalus on the phylloplane, compared to the abundance of these yeasts in the faeces of the two developmental stages, may indicate that the resolution of the culturing methods was too low to detect yeast species that were present in relatively low numbers.

Several yeast species belonging to the genera *Candida, Cryptococcus, Meyerozyma, Debaryomyces* and *Wickerhamomyces* were previously found to be associated with insects (Nguyen et al. 2007; Suh et al. 2004; Ganter 2006). Mutualistic interactions of yeasts associated with beetles often involve aiding in digestion, fermentation and detoxification of food substrates (Ganter 2006). Likewise, since yeast cells are known to bind various metal ions under a wide range of external conditions (Padmavathy 2008), the Ni resistant yeasts proliferating in the gut of *C. clathrata* may aid in lowering the toxicity of this HM to the beetle. On the other hand, a commensalistic symbiosis may exist during which the beetle serve as vector for yeast dispersal (Morais et al. 2006), while its gut provides a nutrient rich habitat which supports yeast growth, with no effect on the beetle's physiology.

2.6.4 Endophytic bacteria

Most of the endophytic bacteria isolated from the leaves of *B. coddii* were found to be representatives of the genus *Bacillus* (Table 2.5). This was not surprizing since the occurrence of *Bacillus* species as endophytes of hyperaccumulators has been reported before. In fact, Barzanti and co-workers (2007) identified several genera of Gram positive bacteria including *Bacillus, Paenibacillus, Leifsonia, Curtobacterium, Microbacterium, Micrococcus,* and *Staphyloccoccus* as endophytes of *Alysum bertolonii,* a Ni hyperaccumulating plant endemic to serpentine outcrops of central Italy.

In agreement with our own findings (Table 2.5), interspecific variation in HM resistance of endophytes was observed by Lodewyckx and co-workers (2002). They characterized the Zn and Cd resistance of endophytes associated with the shoots and roots of the Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calanibaria.* This suggests that different microbial communities exist in different compartments of the plant (Rajkumar et al. 2009). Our own results indicated that different species may prefer different parts of the leaves. Alternatively, the species that are more Ni resistant may produce extracellular Ni chelating compounds effectively decreasing the free Ni ion concentration and thereby allowing other less resistant bacteria to colonize the same space.

This study revealed only two closely related genera of bacteria. These are known for their ability to produce endospores which may have allowed them to survive the surface sterilization. Other endophytic bacteria may have been killed in the process. A molecular technique approach would probably reveal a higher diversity of not only endophytic bacteria, but also fungi. The culturing of endophytes however, creates the opportunity to study the physiology and chemical interactions of these microorganisms.

2.7 Conclusions

Although only relatively few plants originating from a single sampling area were studied, our results indicated that a number of culturable bacteria and fungi were associated with the laboratory reared *B. coddii*. The fungi were found to be resistant to Ni in varying degrees. To obtain a true picture of the microbial diversity associated with these plants more plant samples from different sampling areas should be analysed in future. Nevertheless, the Ni resistance of the fungi suggests that they are continually exposed to Ni and that they have adapted to exploit the leaves of *B. coddii*. We also showed that certain yeast species present on the phylloplane, may be transferred to *C. clathatra'*s gut. Here they may proliferate during passage through the gut. These yeasts showed high resistance to Ni and we hypothesize that the gut of the beetles creates a selective environment for certain Ni tolerant yeasts and that there is a close relationship between the yeasts and the beetle. The next chapter deals with metabolic interactions that may occur between these yeasts and *C. clathatra.*

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Chapter 3

Preliminary studies on the metabolic interactions of yeasts within the gut of *Chrysolina clathrata*

3.1 Introduction

Chrysolina clathrata is a phytophagous beetle known to feed on the leaves of the nickel (Ni) hyperaccumulator, *Berkheya coddii* (Mesjasz-Przybyłowicz and Przybyłowicz 1999, 2001). Evidence suggests a symbiotic relationship between *C. clathrata* and Ni tolerant yeasts occurring in its faeces (Chapter 2). These unicellular fungi, representing the species *Candida intermedia*, *Cryptococcus flavescens, Debaryomyces hansenii, Meyerozyma guilliermondii* and *Wickerhamomyces anomalus* presumably occur within the hindgut of *C. clathrata.* Such gut-inhabiting fungi are known to be essential to the nutrition of many insects however; few studies were conducted focussing on direct relationships between non-pathogenic fungal symbionts and their insect hosts (Suh et al. 2004).

Sasaki and co-workers (1996) hypothesized that yeast-like endosymbionts of the brown planthopper (*Nilaparvata lugens*) could play a role in nitrogen recycling, i.e. conversion of nitrogenous waste products into compounds of nutritional value for the insect. The authors studied uric acid metabolism in this insect and found that yeast-like symbionts, harboured in the mycetocyte within the insect, are in a syntrophic relationship with the animal by aiding in recycling of the nitrogen of uric acid (Sasaki et al. 1996).

Terrestrial insects usually excrete excess nitrogen as uric acid and/or urates, often in combination with urea, pteridines, certain amino acids, and/or uric acid derivatives, such as hypoxanthine, allantoin and allantoic acid (Gullan and Cranston 2010). Uric acid and urates require less water for synthesis compared to ammonia and urea, are non toxic, not highly soluble in water (at least in acidic conditions), and are excreted essentially dry, without causing osmotic problems. Therefore, the excretion of excess nitrogen as uric acid is highly advantageous to terrestrial insects and enables them to conserve water. The Ni tolerant yeasts within the gut of *C. clathrata* may able to utilize uric acid and that a syntrophic relationship exists between these yeasts and the beetle.

In addition to the challenge of excreting excess nitrogen, *C. clathrata* also has to cope with high (40 000 - 76 000 $\mu g.g^{-1}$) and potentially toxic Ni concentrations in its specialized diet of the Ni hyperaccumulator *B. coddii.* The beetle manages to effectively sequestrate Ni in the

faeces through adaptive physiological mechanisms of the Malpighian tubules and the midgut (Migula et al. 2011, Przybyłowicz et al. 2003). Results, presented in Chapter 2 indicated that the yeast strains isolated by us from the beetle's faeces showed resistance to Ni. These yeast strains therefore seem to have come in close contact with Ni in the faeces and have adapted to this heavy metal. We hypothesize that these yeast strains confer some advantage to the beetle by aiding in the sequestration of Ni in the faeces. However, to appropriately test this hypothesis, experiments based on axenic *C. clathrata* colonies are required during which the beetles are fed with leaves inoculated with selected yeast strains, followed by monitoring insect development and analyses of the Ni content of the beetles' faeces. Unfortunately, no such investigations have been conducted and preliminary studies are required to hone experiments with axenic colonies of *C. clathrata.*

In this preliminary study we aimed to explore the potential interactions of yeast strains associated with the gut of *C. clathrata* focussing on nitrogen metabolism and Ni sequestration. During this study we had several objectives: (1) to determine whether it is possible to rear an axenic colony of *C. clathrata,* which is fed with surface sterilized *Berkheya coddii* leaves, and compare the performance of such a colony with that of a control colony fed with non-sterilized leaves containing microbial epiphytes, (2) confirm that uric acid and urea are present in the faeces of *C. clathrata*, (3) determine whether yeast strains originating from the faeces of *C. clathrata* are capable of utilizing uric acid as a sole carbon and/or nitrogen source and (4) if a highly Ni resistant strain of *M. guilliermondii* is capable of sequestering Ni.

3.2 Materials and Methods

3.2.1 Rearing of axenic insects

A method adapted from Charnley and co-workers (1985) was used to rear axenic *C. clathrata* colonies. *Chrysolina clathrata* colonies were reared in Petri dishes placed within 60 L sterile perspex chambers. The chambers were sterilized with 2 % (v/v) peracetic acid (Peratize, Medichem, Atlantis, Western Cape, South Africa). Air, pumped into the chambers at a rate of 1 $L.min^{-1}$, through a sterile air filter with 0.45 µm pores (Sartorius). A natural photoperiodic regime of 12 h light and 12 h dark was followed and humidity was maintained by placing sterile water in a glass container, upside down in a Petri dish. However, controlling the

humidity in the chambers was not possible due to the destabilizing effect of regularly adding fresh leaves to feed the beetles. Two colonies were reared in separate chambers, one of which served as the control chamber, while the other was used as the experimental chamber.

Axenic larvae were produced using the method described by Charnley et al. (1985). Four day old eggs obtained from laboratory-reared colonies of *C. clathrata* (Chapter 2) were washed with sterile distilled water to remove any debris on the egg surface. Misshapen or discoloured eggs were discarded as potential reservoirs of bacteria. A batch of 20 beetle eggs was surface-sterilized by placing them in 2 % peracetic acid for 20 min followed by rinsing them in sterile distilled water. The rinsed eggs were then transferred to nutrient agar (NA; Biolab, Merck, Wadeville, Gauteng, South Africa) plates and incubated at 30 °C to allow larvae to hatch. The plates were examined daily for microbial growth and contaminated eggs were discarded.

Upon hatching, the larvae were retained on the agar plates for a further two days. The insects were regarded as axenic if the agar remained free of microbial growth. The plates containing the larvae were then surface sterilized by spraying them with 2 % (v/v) peracetic acid, and after 60 min. they were transferred into the chambers. The colony of beetles reared in the control chamber was fed non-sterile *Berkheya coddii* leaves while the experimental colony was fed leaves of which the surfaces were sterilized using the method described in Chapter 2. Growth and development of the insects were monitored over a period of 30 days.

The sterility of the chambers and insects was tested regularly by inoculating McCartney bottles containing nutrient broth (NB; Biolab, Merck, Wadeville, Gauteng, South Africa) with faecal material and swabs of the chambers. The bottles were subsequently monitored for growth while incubating them for four days at 30 °C.

3.2.2 Determination of uric acid and urea in faeces

3.2.2.1 Sample preparation

To detect uric acid and urea in the faeces, a UPLC^{TM}-MS (Ultra Performance Liquid Chromatography Mass Spectrometry) and colorimetric method was used respectively.

Faeces were collected from the Petri dishes in which the beetle colonies were reared. Subsequently, 0.1 g of the faeces was suspended in 1 ml distilled water to extract any urea present in the faeces. A similar procedure was used to extract uric acid. However, due to the low solubility of uric acid in water, the faeces were suspended in 2 mM ammonium hydroxide (NH_4OH). All extracts were vortexed for 1 min. The resulting suspensions were purified using reverse phase separation columns (Supelco SPE 18; Sigma-Aldrich, GmbH, Steinheim, Germany) that were activated with 50 µl methanol, washed with 50 µl distilled water and dried prior to adding the suspension. The purified faecal extract solutions were subsequently analysed.

3.2.2.2 Detection of uric acid in faeces using UPLC™-MS

A volume of 20 µl purified extract solution was introduced into a Waters Xevo TQ MS containing a Phenomenex Kinetex 2.6 u HILIC column (150 x 2.1 mm). Uric acid was eluted at 0.25 ml.min^{-1} from the column using the Waters Acquity UPLC method with a mixture of 10 mM ammonium acetate and 10 mM ammonium acetate in H_2O:acetonitrile (25:475). The retention window was set at 14 min. Mass spectrometry analysis was conducted using a triple quadrupole mass spectrometer (Waters Xevo) by positive electrospray ionization (ESI+) in multiple reaction monitoring mode (MRM). Uric acid (Sigma-Aldrich, GmbH, Steinheim, Germany), with a retention time of ca. 5.3 min., was used as standard.

3.2.2.3 Colorimetric procedure for detecting urea in faeces

The Mulvaney and Bremner (1979) colorimetric method for the determination of urea as described by Carlile and Dickinson (1997), was adapted to detect urea in the faeces of *C. clathrata*. Urea was determined by pipetting 50 µl of each standard (Merck Wadeville, Gauteng, South Africa) or purified extract along with 50 µl milliQ water and 150 µl of colour reagent into test tubes (5 ml volume). The colour reagent consisted of 5 ml of a 2.5 % aqueous solution of diacetyl monoxime (Sigma-Aldrich, GmbH, Steinheim, Germany) added to 3 ml of a 0.25 % aqueous solution of thiosemicarbazide (Sigma-Aldrich, GmbH, Steinheim, Germany) and diluted to 100 ml with a sulphuric acid/phosphoric acid mixture (20 ml of concentrated H_2SO_4 added to 500 ml, 85 % w/w H_3PO_4 and diluted to 1 L with distilled water). The test tubes were sealed with parafilm and incubated in a water bath with a light proof cover at 85 °C for 30 min. Subsequently the contents of the test tubes were transferred to plastic cuvettes and the absorbance was measured using a BioRad plus spectrophotometer

at a wavelength of 540 nm against a water blank carried through the same procedure. The urea concentration in the faeces was estimated using a calibration curve prepared with reagent grade urea (Sigma-Aldrich, GmbH, Steinheim, Germany).

3.2.3 Yeast growth on uric acid

Selected yeast strains isolated from the faeces of *C. clathrata* as described in Chapter 2 were tested for their ability to grow on uric acid as a sole nitrogen or carbon source. The yeasts strains represented the following species: *Meyerozyma guilliermondii, Cryptococcus flavescens* and *Wickerhamomyces anomalus*. The method adapted from Byzov and co-workers (1993) included the use of the following media, which were autoclaved for 15 min at 121°C: (1) 10 $g.L^{-1}$ uric acid (the only source of carbon), 6.7 $g.L^{-1}$ Yeast Nitrogen Base (YNB) (Difco); (2) 1 $g.L^{-1}$ uric acid (the only source of nitrogen), 11.3 $g.L^{-1}$ Yeast Carbon Base (YCB) (Difco). Media for positive controls were prepared by supplementing 6.7 $g.L^{-1}$ YNB and 11.3 $g.L^{-1}$ YCB with 5.0 $g.L^{-1}$ glucose and 3 $g.L^{-1}$ $(NH_4)_2SO_4$ respectively. The negative control media contained 6.7$g.L^{-1}$ YNB and 11.3 $g.L^{-1}$ YCB without any carbon and nitrogen sources respectively. All media contained 20 $g.L^{-1}$ agar and uric acid was autoclaved separately.

We inoculated test tubes, each containing 5 ml selective yeast medium broth (YM) with the above mentioned yeast strains and incubated the cultures at 26 °C for 24 h on a tissue culture roll drum (10 rev min^{-1}). The yeast cells was separated from the growth media by centrifugation at 15 000 g for 30 s and the supernatant was discarded. The cells were washed twice with distilled water to remove any remaining media and resuspended in physiological saline solution. A serial dilution was made of the cell suspension and each dilution was plated onto the above mentioned defined media. Plates were incubated at 26 °C for 5 days after which the absence or presence of growth was noted.

3.2.4 Nickel sequestration ability of *Meyerozyma guilliermondii*

In Chapter 2 it was shown that the yeast strains representing the species *M. guilliermondii* showed the highest resistance to Ni. *Meyerozyma guilliermondii* CAB 91 was therefore selected to determine the Ni sequestration ability thereof. Yeast cell biomass for Ni uptake experiments was obtained by inoculating 500 ml conical flasks, each containing 100 ml YM

broth, with *M. guilliermondii* CAB 91. The flasks were then incubated at 30 °C for 24 h on an orbital shaker agitated at 150 rev min^{-1}. The resulting biomass was harvested by centrifugation and washed twice with distilled water to remove the growth medium, and subsequently inactivated by heating in an oven at 80 °C for 24 h (Padmavathy 2008).

Nickel uptake experiments were conducted in 500 ml culture flasks containing 100 ml of either 0.5 mM or 1 mM Ni solution prepared by dissolving nickelchloride hexahydrate ($NiCl \cdot 6H_2O$) (Merck) in distilled water. Morpholinoethane sulfonic acid (MES; 5 mM) served as buffer and the pH of the Ni solution was adjusted to 5.5 with 100 mM tetramethylammonium hydroxide solution (Junghans and Straube 1991). After the adsorption process, the cells were collected by centrifugation (15 000 g) and the metal concentration in the supernatant was measured using the dimethylglyoxime colorimetric method described in Chapter 2.

Two cell mass concentrations of 1 $g.L^{-1}$ and 2.5 $g.L^{-1}$ were used to test the effect of cell concentration on Ni uptake. The cell mass concentration was adjusted by measuring the absorbance of cell suspensions at 600 nm and comparing it to a calibration curve prepared with yeast suspensions containing a known mass of yeast cells.

3.3 Results

3.3.1 Axenic *C. clathrata* beetles

Subsequent to the sterilization of the beetle eggs, 16 larvae developed from the batch of 20 eggs. Six of these larvae were used as the control colony (non-sterile) and 10 eggs were used for the experimental axenic colony (EAC). After seven days of rearing the EAC on surface sterilized *B. coddii* leaves, sterility tests of the EAC's faeces showed that the larvae were axenic (Figure 3.1). In contrast, growth was observed in the bottle containing nutrient broth inoculated with the faeces of the control colony.

After nine days of rearing the larvae in the chambers all 6 of the larvae in the control colony were alive, however four larvae of the axenic colony died. Six days later the larvae of the control colony entered the ecdysis phase. One larva died in the control colony whilst 2 larvae died in the axenic colony. The two remaining larvae in the axenic colony failed to enter the

ecdysis phase of development. After one month, since introducing the larvae into the chambers, two larvae of the control colony developed into adult beetles. In contrast, only 1 larva of the axenic colony survived however, the animal never completed its development.

Figure 3.1: Testing of beetle faeces from both the control (marked here as conventional) and experimental colonies confirmed that the chambers were sterile. Growth was clearly visible in the control, while no visible growth was observed in the medium inoculated with faeces from the axenic culture.

3.3.2 Uric acid and urea in faeces

Using UPLC^{TM}-MS we confirmed the presence of uric acid in the faeces of adult *C. clathrata* beetles (Figure 3.2). The retention time (5.32 - 5.34 min.) of the highest peaks in the multiple response measurement (MRM) chromatograms of the faecal extract samples corresponded to the retention time (5.26 min.) of the uric acid standard. Faecal extracts made using NH_4OH

as solvent resulted in a higher uric acid concentration in the extract sample than those extracts performed using water as solvent. This can be seen in the difference in scale between the MRM chromatograms (Figure 3.2).

We were also able to detect urea in *C. clathrata's* faeces using a colorimetric technique. Preliminary data of three replicates indicated that the average urea concentration in purified faecal extracts using NH_4OH as solvent was 85.6 ± 6.7 $g.L^{-1}$. Extracts of the same faecal samples made using distilled water as solvent contained a higher average urea concentration of 127.4 ± 0.94 $g.L^{-1}$).

3.3.3 Yeast growth on uric acid as a sole nitrogen or carbon source

All three yeast strains we tested, which represented both ascomycetous and basidiomycetous yeast species, could utilise uric acid as a sole nitrogen and carbon source (Table 3.1). However, larger yeast colonies were formed on the agar medium containing uric acid as sole carbon source, than on the medium containing uric acid as sole nitrogen source.

Table 3.1: Ability of ascomycetous and basidiomycetous yeast strains, isolated from the faeces of *C. clathrata,* to utilize uric acid as a carbon and/or nitrogen source.

Yeast strains	Classification*	C source	N source
Meyerozyma guilliermondii CAB 91	Ascomycete	+++	+
Cryptococcus flavescens CAB 87	Basidiomycete	+++	+
Wickerhamomyces anomalus CAB 103	Ascomycete	+++	+

+++ indicates well developed colonies (diameter ca.4 mm) plates after 5 days of incubation at 26 °C; + indicates slow developing colonies (diameter ca. 1 mm) plates after 5 days of incubation at 26 °C. *According to Kurtzman and Fell (2006)

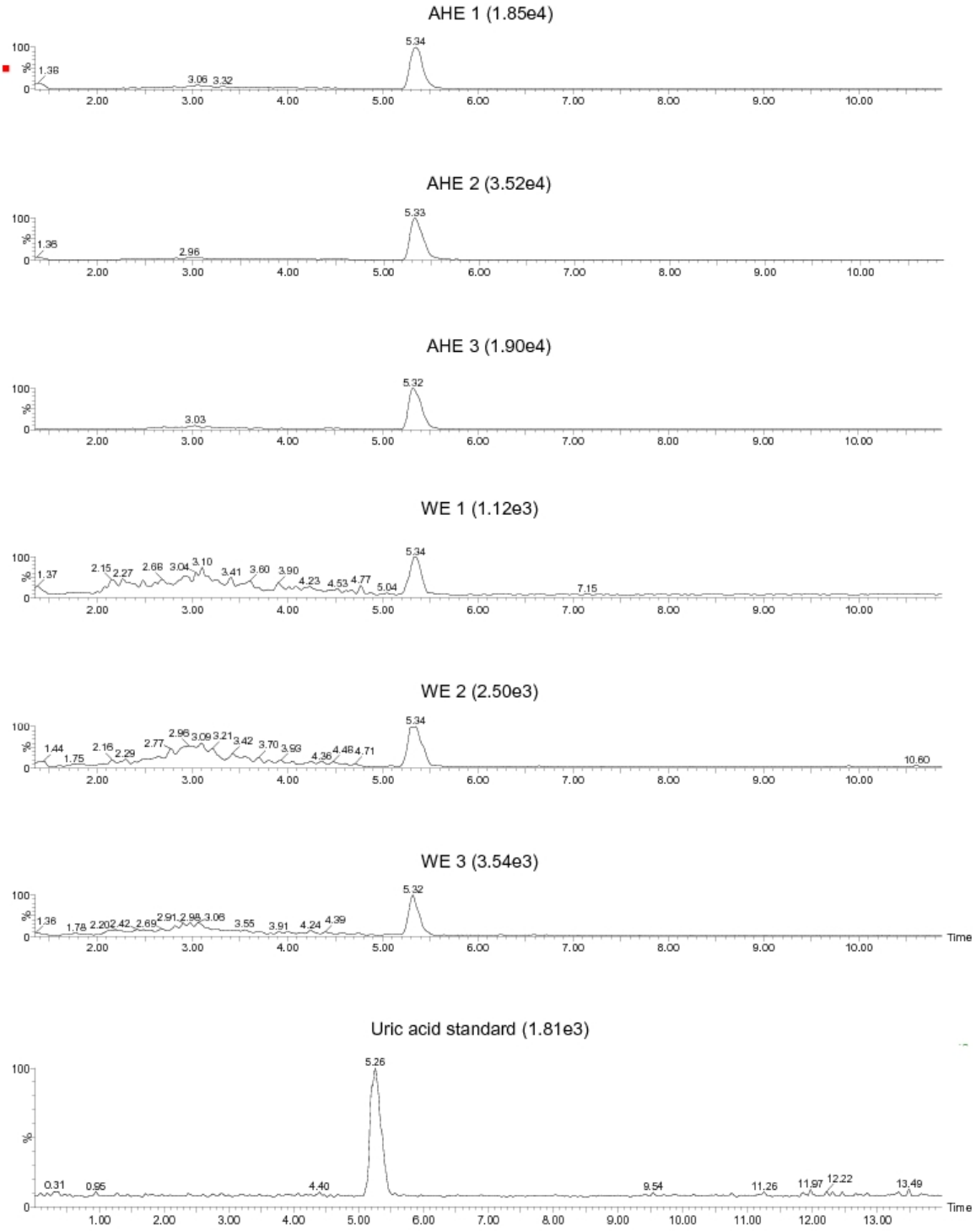

Figure 3.2: UPLC^{IM}-MS multiple reaction monitoring (MRM) chromatograms of purified faecal extracts indicating the presence of uric acid in the faeces. AHE = extraction of uric acid from faeces using NH_4OH; WE = extraction of uric acid from faeces using distilled water. Numbers in parenthesis indicates scale of chromatograms. Three purified faecal samples, each prepared on separate occasions, were analysed for each extraction method.

3.3.4 Nickel sequestration ability of *Meyerozyma guilliermondii*

Preliminary data showed that *M. guilliermondii* CAB 91 biomass adsorbed Ni from the buffered solutions (Table 3.2). The highest adsorbtion (8.12 $mg.g^{-1}$) was observed with 2.5 $g.L^{-1}$ yeast biomass in a 0.5 mM Ni solution. Adsorbtion of Ni in a 1 mM Ni solution (5.8 $mg.g^{-1}$) was the same irrespective of biomass concentration. The lowest adsorbtion (2.32 $mg.g^{-1}$) was observed when the biomass concentration in a 0.5 mM Ni solution was 1 $g.L^{-1}$.

Table 3.2: Adsorbtion of Ni by *M. guilliermondii* CAB 91 at pH 5.5, 30 °C, with a contact time of 1 h

Yeast biomass $g.L^{-1}$	**1**		**2.5**	
[Ni] mM	**0.5**	**1**	**0.5**	**1**
Ni adsorbsion $mg.g^{-1}$	2.32	5.8	8.12	5.8

3.4 Discussion

3.4.1 Axenic *C. clathrata* beetles

In this preliminary study we were able to rear axenic *C. clathrata* larvae from surface sterilized eggs. However, the mortality rate among these beetles was high and none of the larvae developed past the larval stage. In contrast, two of the six eggs in the control colony developed into adults, indicating that the environment within the rearing chambers of the control colony was more favourable for the development of *C. clathrata* than the environment within the rearing chambers of the experimental colony. The only difference between these groups was the diet of the beetles, while the diet of both beetle colonies consisted of fresh leaves of laboratory reared *B. coddii* plants, the axenic colony was fed leaves that were surface sterilized. This indicated that epiphytes, including yeasts, are essential for the development of *C. clathrata* into adult beetles. However, many other factors need to be taken into consideration in future experiments before we can confirm this.

Factors that may influence the development of the beetles include physical factors such as temperature, humidity and photoperiodic regime (Gullan and Cranston 2010). The chemical composition of the surface sterilised leaves used in our experiments may have been detrimental to the beetles. The axenic colony was fed leaves that were surface sterilized using peracetic acid (Chapter 2). The sterilizing agent may still have been present within the leaf tissue even after thoroughly washing the leaves in sterile distilled water. This may have

influenced the development of the larvae of the axenic colony. Other methods attempted by us to sterilize the leaves e.g. irradiation (5 - 20.0 kGy; Hepro, Cape Town, RSA) and autoclaving (121°C; 15 min) proved to be totally ineffective, because of altered texture and chemical composition of the leaves, which rendered it intractable to the insects. Our previous attempts to rear the beetles on an agar medium diet containing extracts of *B. coddii* leaves have also failed. Thus, of all the sterilization methods evaluated by us, our peracetic surface sterilization showed the most potential for future use, since we were able to keep the beetle larvae alive for at least 7 days while feeding them the sterilized leaves.

In future studies we plan to reintroduce epiphytic microorganisms, including yeasts, to surface sterilize leaves before feeding it to the beetles. By comparing the outcome of such experiments with appropriate controls, we will be able to confirm whether the high mortality rate of the axenic *C. clathrata* larvae was due to the sterilizing agent, or the lack of particular epiphytic microorganisms.

3.4.2 Nitrogen metabolism of yeasts in the gut of *C. clathrata*

Many terrestrial insects are known to excrete nitrogenous metabolic waste products in the form of uric acid and to a lesser extent urea (Gullan and Cranston 2010). We showed that this may also be true in the case of *C. clathrata*. However, further experiments should be performed to determine the proportion of the total nitrogen that is excreted by these beetles in the form of uric acid and urea.

In this study we showed that phylogenetically unrelated yeasts strains, isolated from the beetle's faeces, were able to assimilate uric acid as a sole nitrogen and carbon source. The difference in size between yeast colonies growing on media containing uric acid as sole carbon and sole nitrogen source could have been due to the difference in uric acid concentration between the two media. Another explanation can be that these yeast strains are more effective in assimilating uric acid as a sole carbon source, as opposed to a utilizing this compound as sole nitrogen source.

3.4.3 Nickel sequestration ability of *Meyerozyma guilliermondii*

The ability of yeasts to adsorb various heavy metals from a solution (biosorption) has been well studied in the past (Junghans and Straube 1991; Brady et al. 1994; Padmavathy 2008).

Junghans and Straube (1991) showed that biomass of *M. guilliermondii* can adsorb copper reaching concentrations of up to 34 $mg.g^{-1}$ yeast biomass. In this study we found that biomass of a *M. guilliermondii* strain, isolated by us from the faeces of *C. clathrata,* was able to adsorb Ni at 8.12 $mg.g^{-1}$ (Table 3.2). The aim of future studies will be to evaluate the significance of this Ni sequestration capability of *M. guilliermondii* in relation to the symbiotic interactions within the beetle's gut. It is envisaged that experiments with axenic *C. clathrata* colonies, fed with leaves inoculated with selected yeast strains such as *M. guilliermondii* CAB 91, may provide us with an indication of the Ni adsorbsion capabilities of these yeasts within the gut of the beetle. Such experiments will include comparisons of the Ni concentrations in the faeces of axenic, control and inoculated beetle colonies. Furthermore, it is possible that living yeast cells may adsorb more Ni than inactivated yeast biomass (Junghans and Straube 1991) and therefore we also plan to test the Ni sequestration ability of metabolically active yeast cells.

3.5 Conclusions

Although we were unable to rear an axenic colony of *C. clathrata* to adulthood during this preliminary study, our study laid the foundations for future experiments with axenic colonies of this beetle. Our findings also provided some evidence that *C. clathrata* may be dependent on epiphytic microorganisms, which include yeasts, for its successful development. Whether the yeasts species we found in the faeces play an important role in the development of *C. clathrata* still needs to be confirmed in future studies with axenic *C. clathrata* colonies.

In support of our hypothesis that yeasts within the gut of *C. clathrata* are able to utilize uric acid, and that a syntrophic relationship exists between these yeasts and the beetle, we found that both uric acid and urea are present in the faeces of this insect. Also, phylogenetically unrelated yeast strains isolated from the beetle's gut were able to assimilate uric acid as sole carbon and nitrogen source.

We found that biomass of *M. guilliermondii* CAB 91 which originated from the gut of *C. clathrata* is capable of sequestering Ni from an aqueous solution, thereby providing the first indications that yeasts may contribute to reducing the toxicity of Ni to *C. clathrata* by

sequestering the HM in the faeces of this beetle. However, more studies with axenic colonies are needed to confirm these preliminary findings.

3.6 References

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Chapter 4

General conclusions and future research

In this study we isolated and identified representatives of various yeast, filamentous fungal and bacterial species, originating from laboratory-reared *B. coddii* plants and *C. clathrata* beetles. Isolating such culturable microorganisms allows for the study of microbial physiology and the manipulation of microbial interactions to better understand symbioses within natural environments. Some progress was made towards understanding the symbioses between *B. coddii, C. clathrata* and nickel (Ni) resistant culturable microorganisms. However; to obtain a better picture of the symbioses occurring *in natura* the culturable microbial diversity of more plant samples from various sampling areas in the natural environment should be analysed. Nevertheless, using the results obtained during this study we may speculate on the role and interactions of some culturable microorganisms, associated with *B. coddii* and *C. clathrata,* within serpentine environments.

4.1 Microorganisms isolated from *B. coddii*

4.1.1 Filamentous fungi

We were successful in isolating representatives of various filamentous fungal genera from the leaves of *B. coddii* (Chapter 2). Some of these fungi are known pathogens of plants and they may have a similar life strategy while ocurring on *B. coddii.* The high nickel (Ni) resistance of these filamentous fungi certainly suggests that they do have a close relationship with *B. coddii* and that they have adapted to exploit this plant. However, the nature of the symbiotic interaction taking place between these fungi and *B. coddii* still needs to be investigated. Koch's postulates can be applied to determine if the fungal strains we isolated are indeed phytopathogenic fungi. Subsequently, the elemental defense hypothesis can be tested by rearing two groups of *B. coddii* plants, one group with high and and one group with low Ni concentrations within their leaves. Inoculations of these plants with pathogenic strains and the subsequent occurrence of disease on either or both plant groups will give us information on the effectiveness of HM hyperaccumulation as a defense mechanism.

4.1.2 Endophytic bacteria

Our discovery of several species of Ni resistant endophytic bacteria, belonging to the genus *Bacillus,* within *B. coddii* (Chapter 2) supports the findings of others studying the endophytes of metal hyperaccumulating plants. Our future studies should focus on interactions between the bacterial endophytes of *B. coddii* and microbial phytopathogens, as well as epiphytes

occurring on the plant. It is possible that the bacterial endophytes may have an antagonistic effect on pathogenic fungi or, the bacteria themselves may become pathogenic under certain conditions. Therefore, the symbioses between *B. coddii* and its bacterial endophytes should also be studied further.

4.1.3 Phylloplane yeasts

The yeast species we encountered on the phylloplane of *B. coddii* are common colonizers of this habitat. Interestingly, the yeast strains we isolated showed low resistance to Ni compared to the yeasts we isolated from *C. clathrata*'s faeces. This indicates that the epiphytic yeasts were probably not continually being exposed to high Ni concentrations, and that their epiphytic habitat contains much less Ni than the environment endophytes of *B. coddii* are exposed to. Future studies may include elucidating the interactions between phylloplane yeasts and *B. coddii,* as well as conducting surveys of the yeast diversity associated with natural stands of *B. coddii* on serpentine outcrops of the Barberton Greenstone Belt (BGB).

4.2 Yeasts associated with the gut of *C. clathrata*

We isolated yeasts from the faeces of *C. clathrata* (Chapter 2) and in preliminary studies (Chapter 3) found indications that these yeasts may be metabolically active within the gut of *C. clathrata.* To prove that these yeasts are localized within the gut of the beetle, dissections of the beetles need to be done and the gut should be plated out onto a yeast selective medium. Alternatively, moleculer techniques can be used to detect the yeasts and perhaps even reveal unculturable endosymbiotic species localized within specialized structures called mycetozomes.

Regarding the interaction of these yeasts with *C. clathrata,* we hypothesized that there may be a mutualistic association were both parties benefit form the interaction. Preliminary efforts to test this hypothesis (Chapter 3) by rearing axenic insects gave encouraging results and these studies will be continued. It is also possible that the yeasts may use *C. clathrata* as a host allowing them to survive unvavourable environmental conditions. This hypothesis can be tested by performing a broader survey on the ecology of yeasts in the BGB over an extended period of time during which parameters such as seasonal changes may be considered.

During this study we observed that the adult beetle's gut selected for specific yeast species that also occurred on the leaves. However, we isolated different yeast species from the larvae. Thus, interesting questions that need to be addressed in future studies are: Does a shift occur in the yeast community and, what is the the effects of such a potential shift on the beetle's ontogeny?

4.3 *B. coddii* and *C. clathrata*: a model system for adaptation and co-evolutionary studies?

Berkheya coddii, C. clathrata and their associated microorganisms are potentially excellent candidates to be used as a model system for adaptation and co-evolutionary studies due to various factors. Firstly, this plant and beetle can be reared with relative ease in the laboratory and large experimental groups can be established. Secondly, the presence of high Ni concentrations in this food web serves as an easily observable and manipulable selective agent. Thirdly, the physiological adaptations of *B. coddii* and *C. clathrata* enabling them to cope with high Ni concentrations have already been elucidated by others allowing us to observe any changes in these physiological structures/mechanisms during experiments. Lastly, there are culturable microorganisms associated with the plant and beetle allowing us to manipulate the interactions of the microorganisms with *B. coddi* and *C. clathrata.*

4.4 In conclusion

We found a variety of culturable endo- and epiphytic microorganisms associated with leaves of the Ni hyperaccumulator *B. coddii.* We also discovered a number of Ni resistant yeast strains, representing different species, in the faeces of *C. clathrata*, an insect herbivore of *B. coddii.* In addition, our preliminary experiments indicated that these yeasts may be in a syntrophic relationship with *C. clathrata* and, by being able to sequester Ni, the yeasts may contribute to reducing the toxicity of Ni to this insect. However, many studies are still needed to fully understand the symbioses between *B. coddii, C. clathrata* and nickel (Ni) resistant microorganisms.